HOST AND PATHOGEN DETERMINANTS OF ALLERGIC AND INVASIVE FUNGAL DISEASES

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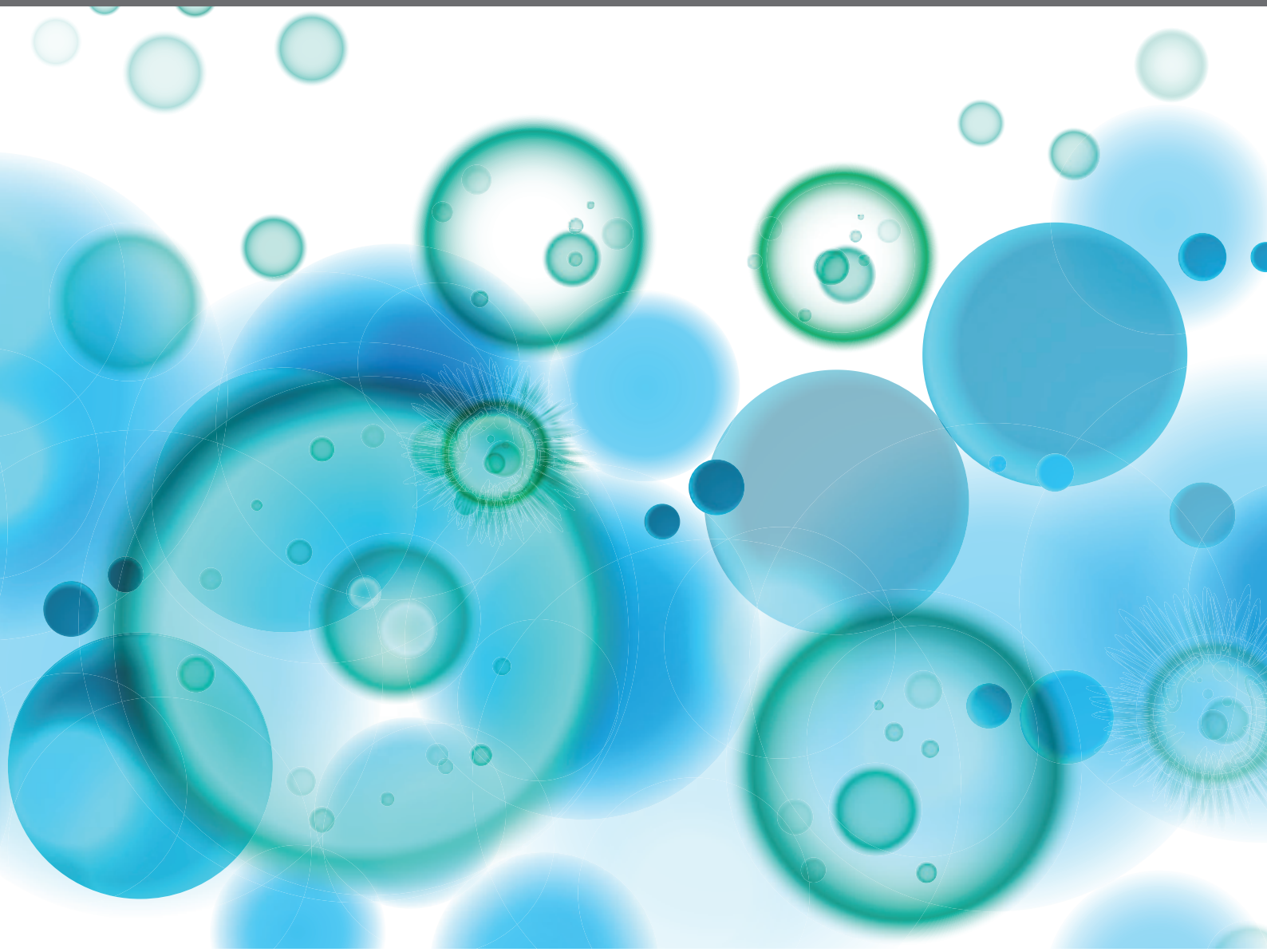

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HOST AND PATHOGEN DETERMINANTS OF ALLERGIC AND INVASIVE FUNGAL DISEASES

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Table of Contents

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EDITORIAL
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Editorial: Host and Pathogen Determinants of Allergic and Invasive Fungal Diseases

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Editorial on the Research Topic

Host and Pathogen Determinants of Allergic and Invasive Fungal Diseases

Fungal diseases are mostly known as opportunistic acute life-threatening invasive fungal diseases, such as *Candida* blood stream infection, invasive aspergillosis, cryptococcal meningitis, *Pneumocystis* pneumoniae, histoplasmosis, and mucormycoses. But they also include chronic fungal infections that are defying treatment, such as mycetoma, sporotrichosis, fungal keratitis and chronic pulmonary aspergillosis, coccidioidomycosis, or histoplasmosis; and fungal allergic diseases, such as allergic bronchopulmonary mycoses, allergic fungal sinusitis, hypersensitivity pneumonitis, or atopic dermatitis. This high heterogeneity in the onset and clinical course of fungal diseases raises fundamental questions about their pathogenesis, which results from either a lack of recognition by the immune system or an excessive inflammatory response. The 10 articles of this themed collection highlight the latest advances regarding host and pathogen determinants of allergic and invasive fungal diseases.

Two critical reviews of the literature update the current state of knowledge on invasive fungal diseases and allergic mycoses. Firstly, Van Dyke et al. address the battle against coccidioidomycosis, a neglected disease whose incidence has raisen to 350,000 cases/year in the desert areas of the western United States. Their review details the current knowledge on the protective host immune responses, potential vaccines, and new treatments against *Coccidioides immitis*. Secondly, Hadebe and Brombacher address the excessive inflammatory response associated with fungal diseases, particularly in the setting of chronic disease. They summarize the current view on (i) early exposure to environmental fungal species; (ii) their contribution to the development of allergic responses; (iii) the mechanisms of host tolerance controlling immune hyper-responsiveness to ubiquitous fungi, and (iv) the scarce knowledge on the early factors involved in the development of fungal allergic diseases. These issues relate to the major unanswered question is why a given fungus (*Aspergillus*, *Alternaria*, *Cladosporium*, *Malassezia,* or *Penicillium*) will cause allergy or infection rather than be perceived as innocuous at a given time point in a given human host. Allergy to fungal molecules triggers allergic bronchopulmonary mycoses, allergic sinusitis, allergic bronchitis, hypersensitivity pneumonitis, and atopic dermatitis. Fungal molecules with established allergenic potential have been previously described, but the tools for *in vitro* or *in vivo* diagnosis

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of fungal allergy remain scarce. One of the most severe chronic forms of fungal disease is allergic bronchopulmonary mycoses, which occur in patients with chronic lung diseases such as COPD or cystic fibrosis. In this collection, Michel et al. demonstrate in 29 adults with cystic fibrosis that basophil and lymphocyte activation tests can enhance the diagnosis of allergic bronchopulmonary mycoses, compared to the usual humoral immunity biomarkers.

Understanding how the immunological machinery of the host and the fungal armory of stress responses interact to dictate the outcome of the host-fungus interaction. Understanding these interactions will provide critical insights into fungal disease mechanisms and pinpointing relevant immune and fungal molecules that ensure protection or drive disease might lead to new therapeutic targets. In this regard, Loureiro et al. explore macrophage extracellular traps (METosis), which are extracellular DNA fibers released by macrophages that are able to entrap and kill various microbes. They observe that upon contact with *C. albicans in vitro*, (i) macrophages phagocytose and engulf yeast cells, (ii) METosis and phagocytosis can occur simultaneously, and (iii) both processes are important in controlling yeast cells proliferation in the first hours of infection, particularly in neutropenic patients (Loureiro et al.). Their findings suggest that yeast extracellular DNase activity might be an important virulence factor, which would degrade the extracellular DNA traps (Loureiro et al.). In another *in vitro* study conducted on *Talaromyces marneffei*, Li et al. unveil a novel pathway by which this opportunistic fungal pathogen may suppress the immune response to its advantage. Their findings show how IL-6, a key factor in acute inflammatory responses, is down-regulated in bronchial epithelial cells infected with *Talaromyces marneffei*. This inhibition occurs via lncSSBP1, a novel long non-coding RNA that specifically interacts with heterogeneous nuclear ribonucleoprotein K, which is involved in IL-6 mRNA processing (Li et al.). Likewise, the two *in vivo* mouse studies published in this collection provide novel insights into host-fungi interactions mechanisms. Amarsaikhan et al. identify a novel role for the adiponectin pathway in the inhibition of lung inflammatory responses to chitin and *A. fumigatus* inhalation. Fa et al. support that TNF-α expression by an engineered *Cryptococcus neoformans* strain is insufficient to drive complete immune protection, but strongly enhance protective responses during primary cryptococcal infection when compared to wild type strain-infected mice.

The incidence of invasive fungal diseases is increasing with a global rise in the number of immunocompromised patients, who have deficient antifungal defense mechanisms that primarily affect the phagocytic activity of immune cells and the overall inflammatory response. Yet, infection by molds and other pathogens induce elevated levels of several cytokines, including IL-6 and IL-8, even in severely immunocompromised patients (Rawlings et al.). In a clinical study, Rawlings et al. find that elevated IL-6 and IL-8 levels in the blood or BAL fluid at the time of bronchoscopy and, perhaps more importantly, rising levels in blood 4 days following bronchoscopy predict mortality in 106 patients with underlying hematological malignancy who underwent bronchoscopy for suspected mold infection.

The high variability in the onset and clinical course of fungal diseases in patients raises fundamental questions about host and fungal factor that are critical in regulating pathogenesis. Exciting approaches aimed at dissecting the respective influence of host and fungus genetic backgrounds on disease severity, including fungal diseases, are rapidly advancing. Puértolas-Balint et al. address the issue of the fungal pathogen-associated factors by comparing the virulence-associated gene (VRG) content in the whole-genome sequence of four *A. fumigatus* isolates from either clinical or environmental origins. They highlight a high genetic diversity among these isolates, with up to 68,352 total number of total genetic variants. In contrast, the genomic VRG content was similar in all isolates, demonstrating that clinical and environmental isolates share the same pathogenic potential, at least at the genome level. However, their comparative genomic analysis highlight the presence of both single nucleotide polymorphisms within VRGs, and repetitive genetic elements located next to VRG groups, which could alter gene regulation and explain heterogeneous virulence phenotypes observed among *A. fumigatus* isolates (Puértolas-Balint et al.). On the flipside of the coin, Danion et al. investigate how the stimulation of neutrophils and peripheral blood mononuclear cells from *STAT3*-deficient patients impacts the immune responses induced by *A. fumigatus*. They find that *STAT3*-deficiency leads to a defective adaptive immune response against *A. fumigatus* infection, characterized by lower IFN-γ and IL-17 responses, and conclude that the potential benefit of IFN-γ treatment in *STAT3*-deficient patients with aspergillosis warrants further study (Danion et al.).

Overall, this themed collection enhances our knowledge of the molecular and cellular processes involved in fungal diseases susceptibility, which paves the way toward personalized medical interventions based on host-directed risk stratification and individualized diagnosis and therapy.

AUTHOR CONTRIBUTIONS

SR drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
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Evaluation of Cellular Responses for the Diagnosis of Allergic Bronchopulmonary Mycosis: A Preliminary Study in Cystic Fibrosis Patients

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Background: Allergic bronchopulmonary mycosis (ABPM) is an underestimated allergic disease due to fungi. Most reported cases are caused by *Aspergillus fumigatus* (Af) and are referred to as allergic bronchopulmonary aspergillosis (ABPA). The main risk factor of ABPA is a history of lung disease, such as cystic fibrosis, asthma, or chronic obstructive pulmonary disease. The main diagnostic criteria for ABPA rely on the evaluation of humoral IgE and IgG responses to Af extracts, although these cannot discriminate Af sensitization and ABPA. Moreover, fungi other than Af have been incriminated. Flow cytometric evaluation of functional responses of basophils and lymphocytes in the context of allergic diseases is gaining momentum.

Objectives: We hypothesized that the detection of functional responses through basophil and lymphocyte activation tests might be useful for ABPM diagnosis. We present here the results of a pilot study comparing the performance of these cellular assays vs. usual diagnostic criteria in a cystic fibrosis (CF) cohort.

Methods: *Ex vivo* basophil activation test (BAT) is a diagnostic tool highlighting an immediate hypersensitivity mechanism against an allergen, e.g., through CD63 upregulation as an indirect measure of degranulation. Lymphocyte stimulation test (LST) relies on the upregulation of activation markers, such as CD69, after incubation with allergen(s), to explain delayed hypersensitivity. These assays were performed with Af, *Penicillium*, and *Alternaria* extracts in 29 adult CF patients.

Results: BAT responses of ABPA patients were higher than those of sensitized or control CF patients. The highest LST result was for a woman who developed ABPA 3 months after the tests, despite the absence of specific IgG and IgE to Af at the time of the initial investigation.

Conclusion: We conclude that basophil and lymphocyte activation tests could enhance the diagnosis of allergic mycosis, compared to usual humoral markers. Further studies with larger cohorts and addressing both mold extracts and mold relevant molecules are needed in order to confirm and extend the application of this personalized medicine approach.

Keywords: basophil activation test, lymphocyte stimulation test, allergic mycosis, cellular tests, cystic fibrosis

INTRODUCTION

Molds are microscopic fungi ubiquitous in the environment. In immunosuppressed patients, they cause localized or systemic infections. What is less well-known outside allergy clinics is that molds are frequent airborne sensitizers involved in allergic diseases, the most frequent and life-threatening being allergic bronchopulmonary mycosis (ABPM). Most reported cases are attributed to *Aspergillus fumigatus* (Af), which are referred to as allergic bronchopulmonary aspergillosis (ABPA). ABPA occurs in patients with a history of chronic lung disease, such as cystic fibrosis (CF), asthma, or chronic obstructive pulmonary disease (1). The current hypothesis is that chronic inflammatory bronchial diseases alter the immune responses by triggering a Th2 immune response instead of an efficient immune clearance following contact with molds (2, 3). Despite several diagnostic criteria updates, the main criterion still relies on the evaluation of humoral IgE and IgG responses to Af extracts, with the shortcoming that these cannot discriminate Af sensitization from ABPA (4). The determination of IgE responses to Af individual proteins with proven allergenicity, commonly referred to as "molecular allergens," improves ABPA diagnostic accuracy (5, 6). Yet, although ABPM was firstly described and most frequently associated with Af, other molds have been documented to trigger allergic pulmonary disease. Their diagnostic criteria are poorly defined and they are infrequently reported in the literature.

The evaluation of the functional cellular responses against allergens is a diagnostic criterion that is currently used in international guidelines (7, 8). *Ex vivo* basophil activation test (BAT) investigates immediate hypersensitivity events whereas lymphocyte stimulation test (LST) explores delayed hypersensitivity. In both tests, whole blood is incubated with serial concentrations of a suspected allergen, followed by flow cytometric quantification of upregulated activation markers (9, 10). Data are scarce for mold-related allergic diseases (11–13). This study aimed to assess the relevance of functional cellular assays for ABPM diagnosis and review *ex vivo* basophil and lymphocyte functional cellular tests in ABPM diagnosis.

PATIENTS AND METHODS

Patients

Adult patients ($n = 29$) followed in our French cystic fibrosis care center and the lung diseases department (Assistance Publique—Hôpitaux de Marseille) were routinely assessed for ABPM diagnosis between April 2017 and January 2018. Patients were categorized in accordance with the final diagnosis as ABPA, Af-sensitized (AF-S), fungal colonization, or control CF patients. These categories were defined as follows: ABPA met all the ISHAM criteria (4); AF-S displayed sIgE to Af (0.1 kUA/L or greater) without fulfilling the ISHAM criteria for ABPA; fungal colonization was defined as at least one filamentous fungus cultured from a bronchial sample during the previous 6 months, without fulfilling the ISHAM criteria, while patients who were categorized in none of the previous categories were considered as control CF patients. Demographic and laboratory data for the study cohort are detailed in **Table 1**.

Functional Cytometric Tests

All the assays were done with Af, *Penicillium notatum* (Pen), and *Alternaria alternata* (Alt) extracts (Bühlmann Laboratories®, Schönenbuch, Switzerland). BAT was performed with the

TABLE 1 | Demographic and laboratory findings of the study cohort.

	Control	Af-sensitized	ABPA	Fungal colonization	Total
n	16	3	3	7	29
Age (years) (median ± 5–95 percentile)	43 (26-61)	35 (27-44)	21 (17-24)	42 (22-62)	38 (18-62)
Male/female	7/9	1/2	0/3	5/2	13/16
Lung transplantation	12/16	3/3	0/3	4/7	19/29
Time since transplantation (median ± 5–95 percentile)	6 (1-19)	7 (4-14)	/	2 (1-10)	6 (1-17)
Bacterial colonization	9/16	1/3	3/3	6/7	19/29
Fungal colonization	7/16	2/3	3/3	6/7	18/29
Chest HRCT abnormality(ies)	14/16	2/3	3/3	7/7	26/29
Total IgE (kIU/L) (median ± 5–95 percentile)	73 (4–333)	72 (41–100)	414 (74–854)	12 (2–26)	94 (2–469)
IgE Af (kUA/L) (median ± 5–95 percentile)	<0.1	1 (0.1–2)	10 (3–19)	<0.1	1 (0–6)
Eosinophils (mm^3) (median ± 5–95 percentile)	321 (107–623)	327 (161–602)	370 (135–775)	319 (149–565)	326 (128–692)

ABPA, Allergic bronchopulmonary aspergillosis; Af, Aspergillus fumigatus; HRCT, High-resolution computed tomography.

Flow2CAST method (Bühlmann Laboratories®), using CCR3 (CD193) and CD63 as basophil identification and activation markers, following the manufacturer's instructions. Positive controls were anti-RFcεI and the bacterial peptide fMLP. For LST, whole blood was incubated in a 96-well plate with RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) and sequential allergen dilution for 24 h under 5% CO_2. Phytohemagglutinin (PHA, Thermo Fisher Scientific), 10 μg/L, was used as a positive control. Each well was harvested and stained with a mix of the following antibodies: PerCP-anti-CD45 (clone 2D1), FITC-anti-CD3 (clone SK7), APC-anti-CD4 (clone SK3), PE-anti-CD8 (clone SK1), and PeCy7-anti-CD69 (clone L78) (BD Biosciences®, San Diego, California). Flow cytometry was performed on a FACS Canto II (Becton Dickinson, Le Pont de Claix, France) and at least 200 basophils per sample were analyzed for BAT and 10,000 lymphocytes for LST.

Data Expression

Data were analyzed using FACS Diva software (TreeStar, Ashland, OR). Specific IgE (sIgE) to Af extract levels were measured with the Thermo Fisher ImmunoCAP platform (Phadia, Thermo Fisher Scientific, Uppsala, Sweden). All the results were expressed as the basophil or lymphocyte stimulation index, which is the ratio between level of activation with the allergen and level of activation with reaction buffer, with a threshold of 2. Statistical analysis was performed with the R statistical software (14). A correlation matrix was calculated using Pearson's correlation. Mean responses of each group were compared via the Student or Kruskal–Wallis test, a two-sided $p < 0.05$ was statistically significant.

Ethics Statement

The study was based on a retrospective review of medical charts and laboratory results. Under the French law, ethics committee approval and patient consent were not required for this type of non-interventional study, provided the patients had received information and retained the right to oppose the use of anonymized medical data (15, 16).

RESULTS

The results of two patients were excluded from the analysis: one patient with an absolute basopenia (excluded from BAT analysis) and one patient with a deep lymphopenia ($<300/mm^3$, excluded from LST analysis). Negative and positive controls were acceptable for BAT (median 5.19%, range 4.16–5.50, and 85.92%, range 44.99–97.85, respectively) and LST [median 1.52% (1.10–1.97) and 65.39% (31.81–86.89) for CD8 LST, mean 1.29% (1.00–1.80) and 68.32% (45.89–85.73) for CD4 LST]. Status of lung transplantation did not influence the level of basophil activation in anti-RFcεI positive control (median 18.64, range 6.48–21.23 and median 18.54, range 11.96–21.90, $p = 0.53$, for transplanted and non-transplanted patients, respectively) and with all the mold extracts (median 1.68, range 0.69–4.28 and median 1.35, range 0.62–2.11, $p = 0.31$ for Pen; 1.35, range 0.66–6.71 and 1.38, range 0.77–20.37, $p = 0.07$ for Af; 1.29, range 0.60–4.48 and 1.27, range 0.72–7.77, $p = 0.44$ for Alt).

Figure 1A shows that 15 patients (56%) had a positive BAT with at least one mold extract: 10 BAT Af, 6 BAT Pen, and 4 BAT Alt positive. Three patients and one patient had a positive BAT with two or all the three extracts, respectively. BAT showed low levels of activation in controls, in Af-sensitized patients, and in mold-colonized patients. The ABPA group, in which no patient was not lung transplanted, responded with higher basophil activation to Af as compared to other groups, but statistical significance was not reached due to the small sample size (three patients, $p = 0.066$). Af-induced basophil activation in these patients was higher than responses to Pen and Alt (mean 18.48, range 17.00–21.58 for Af vs. 1.02, range 0.54–1.69 for Pen and 0.97, range 0.60–1.21 for Alt), although the significance level was not reached either ($p = 0.066$). These patients had no positive responses with Pen and Alt extracts. When specific IgE to Af was positive (>0.10 kUA/L), BAT Af was significantly higher than BAT Pen and Alt ($p = 0.008$).

Figures 1B,C describes results of lymphocyte activation, which was an infrequent finding with any mold extract and in all patient groups: mean for CD8 LST 3.40 (0.99–7.31), 3.26 (2.84–3.67), 1.87 (1.12–2.67), and 1.82 (1.04–3.38); mean for CD4 LST 1.71 (0.57–3.17), 3.39 (1.76–5.02), 1.23 (0.84–1.49), and 1.50 (0.50–2.69) in control, AF-S, ABPA, and fungal colonization groups, respectively.

Levels of CD4 and CD8 LST did not differ as a function of lung transplantation status or with the level of specific IgE to Af (data not shown). However, the only patient who displayed a strongly positive CD8 and CD4 LST with all the extracts is worth of notice. This 29-year-old woman, lung transplanted with no detectable sIgE to Af presented during the initial investigation with pulmonary micronodules, mucus plugging, and ground glass appearance (thickening and impaction of bronchioles) as evidenced by high-resolution computed tomography chest. Despite this evocative presentation, ABPA was not diagnosed because of the absence of humoral IgE and IgG responses to Af. However, she developed an ABPA 3 months after.

Correlation Analysis

The correlation matrix at **Figure 1D** showed a strong positive correlation of both CD4 and CD8 responses to each mold extract (correlation coefficient r-values ranging from 0.56 to 0.92, $p < 0.001$). In contrast, neither basophil responses to distinct mold extracts nor basophil and lymphocyte responses to a given mold extract were statistically significantly correlated (r-values ranging from −0.24 to 0.09).

DISCUSSION AND REVIEW

Fungal species demonstrated as triggers of allergic pulmonary diseases have been reviewed previously (17, 18), while cellular assays for ABPM diagnosis were reported as early as 1977, as described in **Table 2** (19–22, 25). BAT can demonstrate an immediate-hypersensitivity mechanism (8, 9). Only three studies have evaluated the utility of BAT for ABPA diagnosis so far. All focused on CF patients, a background associated with the highest incidence of ABPA (26). Mirković et al. showed

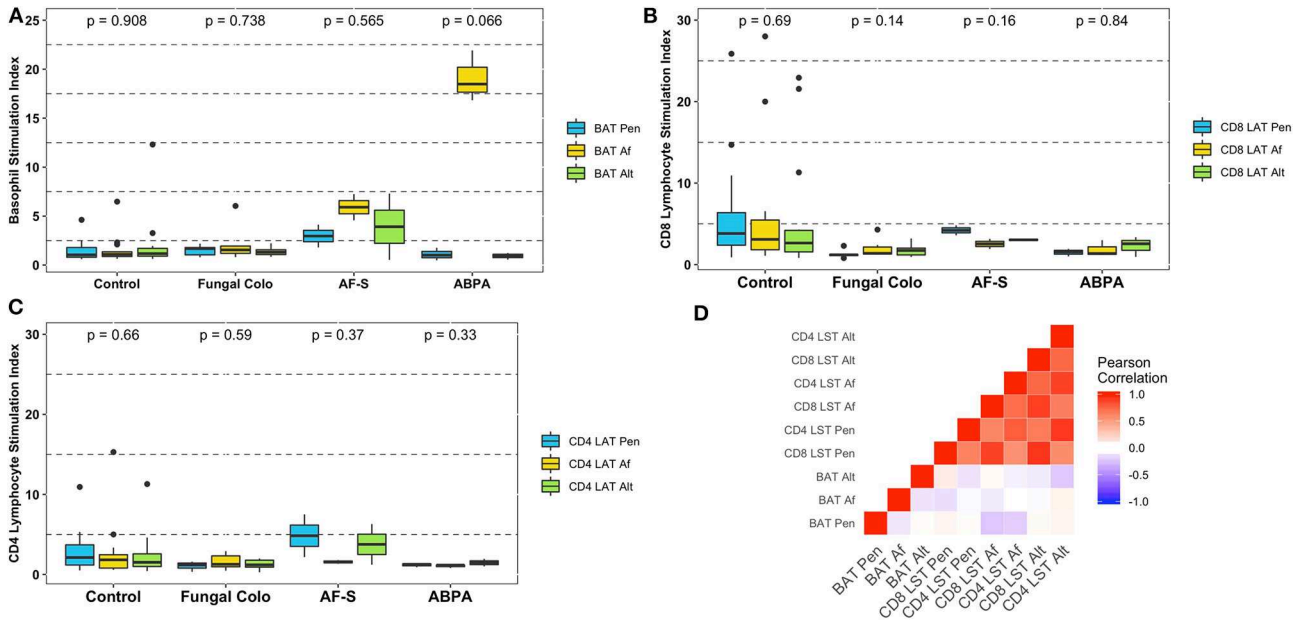

FIGURE 1 | Results of the functional cytometric tests with mold extracts expressed as the stimulation index: BAT results **(A)**, T CD8 LST results **(B)**, T CD4 LST results **(C)**, and correlation matrix of functional cytometric tests with mold extracts **(D)**. *p*-value of the Kruskal–Wallis test, which compares results from Pen, Af, and Alt extracts, is written above each group of patients. ABPA, Allergic bronchopulmonary aspergillosis; Af, *Aspergillus fumigatus* extract; AF-S, Af-sensitized patients; Alt, Alternaria extract; BAT, Basophil activation test; Control, Control patients (without any *Aspergillus*-related disease); Fungal Colo, Fungal colonized-patients; LST, Lymphocyte stimulation test; Pen: *Penicillium* extract.

that BAT with Af extract identified sensitized patients and suggested that a combination of BAT and routine workup could detect ABPA. Moreover, the level of basophil activation was correlated with decreased lung function tests, suggesting that BAT could be used not only as a diagnostic assay, but also as a prognostic marker. Gernez et al. confirmed BAT as an effective and robust diagnostic assay for ABPA, although in their hands, in a contradictory manner, it could not discriminate between Af-sensitized and ABPA CF patients. Katelari et al. proposed the BAT cutoffs of 60.30 and 76.86% of CD63 and CD203c basophil activation for ABPA diagnosis with good sensitivity and specificity. In our patients, BAT with Af extract discriminated Af sensitized from ABPA patients by using a higher threshold. Thus, BAT with fungal antigens is a promising diagnostic tool for ABPM, but further studies are needed to prove the suitability in non-CF patients and by using other fungal antigens.

Evaluation of the lymphocyte responses to allergen is a useful delayed drug hypersensitivity diagnostic tool (10, 27–29). LST has long been used as a diagnostic tool for delayed hypersensitivity against several fungi (**Table 2**). The incorporation of tritiated thymidine was the gold standard for the detection of fungal-specific T cells. Owing to the constraints inherent to radioactive methods, new markers were developed, with CD69 and CD25 upregulation being the most popular (30–32). Currently, an alternative is the lymphocyte proliferation test with 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE) (33). Few studies have evaluated T helper (Th) 2 cytokine production after fungal stimulation, notably IL-5, for ABPM diagnosis (23, 24). Stimulation with Af extract and recombinant proteins in ABPA patients has shown an unusual Th2 immune response (34). Patients with invasive aspergillosis displayed a Th1 phenotype (35) and a Th17 phenotype (36), corresponding to a relevant anti-infectious immune activation. Taken together, these studies show that the immune skew present in a given patient's responses to fungal antigens can be evidenced through functional tests. However, the specificity of Th2 activation induced by fungal antigens as a diagnostic marker for ABPM has not been established, restricting the relevance of this method for ABPM diagnosis. Recently, a new method was developed, based on magnetic antigen-reactive T cell enrichment (ARTE) of $CD154^+$ ($CD40L^+$) T cells, which allowed the identification of rare populations of antigen-specific T cells. Briefly, after PBMC and antigen coculture, antigen-specific cells were sorted via magnetic beads, separating CD154+ conventional T (Tcon) cells from $CD137^+$ $CD154^-$ regulatory T cells. Magnetic enrichment allowed an easy and detailed flow cytometry analysis of subpopulations (37). Tcon cells from lung immunocompromised patients showed a strong Th2 activation after fungal antigen stimulation (38). In our study, LST was not performant for ABPM diagnosis. Yet, our results suggest that LST might predict the development of ABPM. Indeed, the patient with the highest results in our cohort, presenting with a strong CD4 and CD8 T cell activation to fungal extracts, developed an ABPA 3 months after the abnormal LST assay. Detection of peripheral fungal-specific T

TABLE 2 | Overview of reports on cellular assays as a diagnostic tool for allergic mycosis.

References	Population	Allergen(s)	Markers	Main conclusions
Hollmann et al. (19)	79 *C. albicans*-colonized patients 30 healthy controls	*C. albicans*	LST with ^{3}H-thymidine	57% of *C. albicans*-colonized patients show lymphocyte response
Brunet et al. (20)	60 *C. albicans*-delayed HS patients	*C. albicans*	LST with CD25 and CD69	All the patients have CD25 T cells Only patients with syndromic reactions have CD69 T cells
Tsushima et al. (21)	7 patients	*L. aggregatum*	LST with ^{3}H-thymidine	LST is positive in peripheral blood and BAL for all patients Diagnosis of HS pneumonitis induced by *L. aggregatum*
Yoshikawa et al. (22)	One case report	*P. citrinum*	LST with ^{3}H-thymidine	LST is positive in peripheral blood and BAL Diagnosis of HS pneumonitis induced by *P. citrinum*
Matsuno et al. (23)	One case report	*A. alternata* *A. fumigatus* *C. albicans*	LST with ^{3}H-thymidine Evaluation of IL-5 production	LST are positive with *A. fumigatus* and *C. albicans* Only *C. albicans* culture produce IL-5 *in vitro* Diagnosis of acute eosinophilic pneumonia caused by *C. albicans*
Luong et al. (24)	10 allergic fungal rhinosinusitis 11 healthy controls	*A. alternata* *A. fumigatus* *C. herbarum* *P. notatum*	LST with evaluation of cytokine production after 72 h of incubation	Fungal antigens stimulate T-cell activation, with a Th2 immune response (IL-4 and IL-5 production)
Ogawa et al. (25)	Two case reports	*S. commune*	LST with ^{3}H-thymidine	LST is positive in peripheral blood Diagnosis between *Schizophyllum*-asthma and ABPM
Mirkovic et al. (12)	48 CF patients 11 healthy controls	*A. fumigatus*	BAT with CD203c	BAT discriminates non-sensitized and Af-sensitized patients BAT is inversely correlated with FEV1 Antifungal therapy does not altered BAT results
Gernez et al. (11)	74 CF patients 2 asthmatic patients	*A. fumigatus* Asp f 1	BAT with CD203c and CD63	CD203c shows better discriminated performance than CD63 BAT with Af discriminates ABPA and no-ABPA CF patients BAT with Af does not discriminate non-sensitized and Af-sensitized patients BAT with Asp f 1 discriminates ABPA and no-ABPA CF patients
Katelari et al. (13)	56 CF patients	*A. alternata* *A. fumigatus*	BAT with CD203c and CD63	BAT with Af discriminates ABPA and no-ABPA CF patients with a high threshold BAT with Af discriminates Af-sensitized patients who run a higher risk of ABPA No correlation between BAT with Af and BAT with A. alternata

A. alternata, Alternaria alternata; ABPM, Allergic bronchopulmonary mycosis; B. adusta, Bjerkandera adusta; BAL, Bronchoalveolar lavage; C. albicans, Candida albicans; C. herbarium, Cladosporium herbarum; CF, Cystic fibrosis; FEV1, Forced expiratory volume in 1 s; HS, Hypersensitivity; L. aggregatum, Lyophyllum aggregatum; P. citrinum, Penicillium citrinum; P. notatum, Penicillium notatum; S. commune, Schizophyllum commune.

cell, which can activate downstream humoral responses, may highlight a pathological process underlying subclinical ABPA with the potential of development of full-blown ABPA. A larger study with an extended follow-up will be essential to confirm this hypothesis.

Ex vivo cellular activation against several fungi also suggests that ABPM is the result of molecular epitope spreading due to similar T cell activation against three distinct fungi. Watai et al. showed that *de novo* sensitization to fungal antigens is constant during life, contrary to most inhalant allergens (39). Our data showed a major correlation in LST results with all the three allergens, thus an LST cross-reactivity due to a permanent sensitization addition on the T cell repertoire in CF patients.

CONCLUSION

Functional cellular assays are emerging biomarkers for the diagnosis of allergic mycoses. Whereas, IgE and IgG sensitization, which may result from a normal contact with airborne environmental molds, are indirect biomarkers with insufficient specificity, the *ex vivo* functional cellular activity is a direct

marker of *in vivo* mechanisms. Because basophils are involved in lung tissue damage, a strong *ex vivo* basophil activation might not only be an indirect marker of an IgE-linked immune reaction, but also a direct marker of lung disease. Conversely, because T lymphocytes are involved in the initiation, development, and maintenance of the lung Th2-immune response, a major *ex vivo* T cell activation might occur in patients with sub-clinical disease that possibly would progress to overt ABPM. The major weakness of our study is the low number of ABPA cases, due to the prospective design. The major strength is the investigation by means of innovative diagnostic tools.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The study was based on a retrospective review of medical charts and laboratory results. Under the French law, ethics committee approval and patient consent were not required for this type of non-interventional study, provided the patients had received information and retained the right to oppose the use of anonymized medical data (15, 16).

AUTHOR CONTRIBUTIONS

MM, CG, MR-G, and JV contributed conception and design of the study. CG performed the experiments. YS and CCh organized the database. MG, PB, and SP performed the statistical analysis. MM wrote the first draft of the manuscript. CCa, SR, and J-LM wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Aspergillus fumigatus Infection in Humans With STAT3-Deficiency Is Associated With Defective Interferon-Gamma and Th17 Responses

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In humans, loss-of-function mutation in the *Signal Transducer and Activator of Transcription 3 (STAT3)* gene is frequently associated with susceptibility to bacterial as well as fungal infections including aspergillosis, although its pathogenesis remains largely unknown. In the present study, we investigated the immune responses obtained after stimulation with *Aspergillus fumigatus* in STAT3-deficient patients. *A. fumigatus* conidial killing efficiencies of both monocytes and neutrophils isolated from whole blood samples of STAT3-deficient patients were not different compared to those of healthy controls. After stimulation with *A. fumigatus* conidia, lower concentrations of adaptive cytokines (IFN-γ, IL-17 and IL-22) were secreted by peripheral blood mononuclear cells from STAT3-deficient patients compared to those from healthy controls. Moreover, the frequency of IFN-γ and IL-17 producing CD4+ T cells was lower in STAT3-deficient patients vs. healthy controls. Among the STAT3-deficient patients, those with aspergillosis showed further lower secretion of IFN-γ upon stimulation of their PBMCs with *A. fumigatus* conidia compared to the patients without aspergillosis. Together, our

study indicated that STAT3-deficiency leads to a defective adaptive immune response against *A. fumigatus* infection, particularly with a lower IFN-γ and IL-17 responses in those with aspergillosis, suggesting potential therapeutic benefit of recombinant IFN-γ in STAT3-deficient patients with aspergillosis.

Keywords: signal transducer and activator of transcription 3 (*STAT3*), loss-of-function mutation, aspergillosis, innate/adaptive immunity, autosomal dominant hyper-IgE syndrome (AD-HIES), *Aspergillus*, IgE, IgG

INTRODUCTION

In humans, *Signal transducer and activator of transcription 3* (*STAT3*) encodes for a transcriptional regulator, and is a member of the STAT-protein family. STAT3-protein is activated notably by IL-6 family cytokines that signal through gp130 receptors (1), and activated protein has been considered to be an important signal transducer as it evokes distinct responses in different cells (1, 2). STAT3-protein plays a key role in controlling inflammation and immunity, particularly by regulating the expression of acute phase effector-elements (3, 4). Loss-of-function mutations in the *STAT3* gene (STAT3-deficiency) leads to autosomal dominant hyper-immunoglobulin E syndrome (AD-HIES), a primary human immunodeficiency (5, 6). Immunopathology associated with STAT3-deficiency is complex, as this protein is involved in several immunological processes.

STAT3-deficiency has been reported to increase the susceptibility to microbial infections of the skin and lungs, in addition to multisystem disease including cutaneous involvement and developmental defects (5). Susceptibility of the patients harboring *STAT3* mutation to infections by *Staphylococcus aureus or Candida albicans* has previously been investigated (7); although antimicrobial activity of the neutrophils from STAT3-deficient patients were comparable to that from healthy individuals, they displayed a lower production of the cytokines IFN-γ and IL-17, defective production of CXCL8 and antimicrobial peptides (BD2 and BD3) by epithelial cells (8, 9). STAT3-deficient patients showed an increased susceptibility to pulmonary aspergillosis, especially when they had preexisting lung cavities (10). Analysis of the French National Cohort of 74 patients with STAT3-deficiency indicated that 13 (18%) of them had developed at least one episode of pulmonary aspergillosis (11); these episodes were either chronic [aspergilloma and chronic cavitary pulmonary aspergillosis (CCPA)], allergic (allergic bronchopulmonary aspergillosis, ABPA) or mixed forms. However, the immunological defects associated with aspergillosis in STAT3-deficient patients remain unknown.

The objective of our study was to investigate the immune defects associated with STAT3-deficiency upon encountering conidia, the asexual spores which act as the infectious morphotype produced by the ubiquitous fungal pathogen *Aspergillus fumigatus*. We investigated conidial phagocytosis-killing efficiencies of neutrophils and monocytes, and innate and adaptive immune responses of peripheral blood mononuclear cells (PBMCs) isolated from STAT3-deficient patient-blood samples. Moreover, we compared anti-conidial responses of PBMCs isolated from STAT3-deficient patients with and without existing aspergillosis. Our major observation was that STAT3-deficiency is associated with lower adaptive immune responses against *A. fumigatus* infection.

MATERIALS AND METHODS

Patients, Their Blood/Serum Samples

STAT3-deficient patients are followed in France by the Centre de Référence des Deficits Immunitaires Héréditaires (CEREDIH, Paris, France). We first collected sera from 32 patients with STAT3-deficiency to study IgE and IgG responses. We then included 12 STAT3-deficient patients, followed at Necker-Enfants Malades University Hospital, Paris France, for immunological study. Institutional review board approval was obtained (Comité de Protection des Personnes Ile de France 2, France, May 4th, 2015) and written consent was obtained from all the patients included in this study. Control samples were obtained from healthy donors [Etablissement Francais du Sang (EFS), Paris, France, habilitation HS-2015-25101].

Sera from patients with chronic pulmonary aspergillosis (CPA, $n = 10$; four patients had sarcoidosis, one lung cancer, one chronic obstructive pulmonary disease (COPD) and one sequelae following acute respiratory distress syndrome; underlying diseases were not known for the others) and allergic bronchopulmonary aspergillosis (ABPA, $n = 11$; four patients had cystic fibrosis and one asthma; for the others, underlying diseases were not known) and patients without STAT3-deficiency treated with substitutive intravenous immunoglobulins ($n = 5$) were recruited from Necker-Enfants Malades Hospital, Paris, and University Hospitals of Rennes and Lille, all in France.

Isolation of Peripheral Blood Mononuclear Cells (PBMC), Monocytes and Neutrophils

PBMCs were separated on Lymphocytes Separation Medium (Eurobio) by density centrifugation of heparinized blood from STAT3-deficient patients or healthy controls, washed two times and re-suspended in RPMI 1640 + GlutaMAX (Gibco) supplemented with 10% of Normal Human Serum (NHS) and 1% of Pen-Strep (Gibco). PBMC count was determined using LUNA Automated Cell Counter with fluorescent dye to determine absolute number of live cells. Monocytes were purified from PBMC by positive-CD14 selection using CD14 MicroBeads with MS MACS columns (MACS, milltenyi biotec) following the protocol of the manufacturer. The purified monocytes were then resuspended in RPMI 1640 + GlutaMAX supplemented with 10% of NHS or autologous serum and 1% of Pen-Strep, and counted. Neutrophils were purified form the whole blood samples of STAT3-deficient patients and healthy controls

using Neutrophil Isolation Kit (EasySep, Stemcell technologies) following the protocol provided by manufacturer; isolated neutrophils were re-suspended in RPMI 1640 + GlutaMAX with 0.5% NHS, and counted.

A. fumigatus Conidia

A. fumigatus strain used in this study was CEA17ΔakuB^{KU80} that originates from the clinical isolate, CBS 144–89 (12). This strain was maintained on 2% malt-agar slants at ambient temperature. Conidia were harvested from 12 to 15-day old slants using 0.05% Tween–water, washed three times and resuspended in 0.05% Tween–water and then counted using LUNA Automated Cell Counter.

FITC-Labeled Conidia

Conidia were incubated with fluorescein isothiocyanate (FITC; 0.1 mg/mL) in carbonate buffer (0.1 M, pH = 9) at 37°C in a shaken incubator for 1 h, washed three times with carbonate buffer and suspended in phosphate buffered saline (PBS).

Para-formaldehyde (PFA) Fixed Swollen Conidia

Swollen conidia were obtained upon incubating 1×10^8 dormant conidia strain in 50 mL RPMI at 37°C in a shaken incubator for 5 h. Swollen morphotype was verified by microscopy before collecting and washing with water. Conidia were then mildly sonicated to separate aggregates and then *para*-formaldehyde (PFA) fixed overnight at 4°C. Conidia were then washed three times with 0.1 M NH_4Cl, one time with PBS, re-suspended in 1 mL of PBS and conserved at 4°C in aliquots for PBMC stimulation experiments. At least three different batches of fixed swollen conidial samples were used for PBMC stimulation experiments.

Phagocytosis by Monocytes

After isolation, monocytes seeded in 96-well cell-culture plate (2×10^5 per well) were added with 2×10^5 FITC-labeled conidia suspended in RPMI 1640 culture medium containing 10% NHS and 1% Pen-Strep (total culture volume per well, 200 μL). After 1 h incubation, culture medium was removed; Calcofluor White (CFW; 5 μg/mL) in 200 μL of RPMI was added into each well to stain the non-phagocytosed conidia and incubated at room temperature for 15 min. After removing the media, monocytes were fixed with PFA (2.5%) overnight at 4°C. Phagocytosis was evaluated by counting intracellular (FITC only; green) and extracellular (CFW-labeled; blue) conidia under microscope (EVOS cell imaging system, Thermo Fisher Scientific) (13). At least 100 conidia were counted, in triplicate, and phagocytic percentage was expressed as a ratio between phagocytosed conidia out of all conidia counted.

Conidial Killing Experiments

(i) *By monocytes* – performed using conidia of *A. fumigatus* parental strain, CEA17ΔakuB^{KU80}. Monocytes (2×10^5/ well) were incubated with 200 μL of culture medium containing 4×10^4 conidia in 96 well culture-plates. After 3 h of co-incubation, wells were washed with PBS (3×) and fresh culture medium was added. After 8 h of co-incubation, supernatant was removed, wells were washed and cells were lysed with 400 μL of cold water. Media were collected and conidia were spread over Sabouraud-agar plate. Colony forming units (CFU) on the plates were counted after 36 h of growth at 37°C. Experiments were performed in triplicate. (ii) *By neutrophils*: Neutrophils [2.5×10^4 (for MOI 0.5), 5×10^4 (MOI 1), and 10×10^4 (MOI 2)] were incubated with 5×10^4 conidia for 15 h at 37°C in 200 μL of RPMI 1640 + GlutaMAX (Gibco) supplemented with 1% of Pen Strep and 0.5% NHS (Gibco). Conidia of CEA17ΔakuB^{KU80} strain were un-opsonized or opsonized with 20% of NHS in HEPES buffer for 30 min before adding to neutrophils. After co-incubation, supernatant was removed. Cells were lysed with cold water for 20 min and inhibition of germination (conidial killing) was evaluated with resazurin method (14, 15), a colorimetric assay that measures metabolically active fungus. Briefly, 30 μL of resazurin (0.1 mg/mL) was added with 100 μL of RPMI, incubated at 37°C for 48 h and the optical density (OD) was measured at 600 nm. If the fungus is metabolically active (alive), then resazurin changes from blue to pink in color due to the conversion of resazurin into resorufin (7-Hydroxy-3H-phenoxazin-3-one); the percentage of growth was evaluated measuring OD of the sample and comparing with that of positive control (live conidia not co-incubated with neutrophils) and from a negative control (without fungus or cells).

PBMC Stimulation With *A. fumigatus*

To the PBMC (2×10^5/well in 100 μL complete culture medium containing RPMI 1640 + GlutaMAX containing 10% NHS and 1% of Pen-Strep) in a 96-well culture plate added swollen-fixed conidia (2×10^5 in 100 μL complete culture medium); culture medium alone was added to the control wells. Culture plates were then incubated at 37°C in a CO_2 incubator for 1 or 5 days. Following, supernatant from the wells were collected and analyzed for cytokines (one-day culture supernatant for TNF-α, IL-1β, IL-6, IFN-γ, IL-8, and IL-10 and five-days culture supernatant for IFN-γ, IL-4, IL-5, IL-17A, IL-22, IL-6, IL-10) using ELISA duo-SET® (R&D systems). After five-days culture, cells were collected, washed two times with RPMI, suspended in complete culture medium to have a cell count of 5×10^5/mL and stimulated with phorbol myristate acetate (PMA) (50 ng/mL/0.5 million cells) and ionomycin (500 ng/mL/0.5 million cells), along with GolgiStop for 4 h (16). For the analysis of CD4 T-cell polarization (Th1, Th2, Th17, and Treg), surface staining was performed with fluorescence-conjugated MAbs to CD4, CD127, and CD25. Fixable viable dye was used to exclude dead cells. Cells were then fixed, permeabilized using an intracellular staining kit (eBioscience), and incubated at room temperature with fluorescence-conjugated MAbs to FoxP3, IFN-γ, IL-4, and IL-17A. Samples were processed further for flow cytometric analyses (LSR II, BD Biosciences). FITC-labeled anti-human IFN-γ, CD25-FITC, IL4-APC, CD127, BV421 antibodies were purchased from BD Biosciences; IL-17-PE and Fixable Viability Dye eFluor 506 from eBioscience; CD4-PerCP/cyanine5.5 from BioLegend; FoxP3-APC from Invitrogen. Data were analyzed by BD FACS DIVA (BD Biosciences).

Immunoglobulin Quantification

Total IgE and specific anti-*Aspergillus fumigatus* IgE (m3) were measured using immunoCAP® (Thermo Fisher Scientific) following manufacturer's instructions. Specific IgG were determined using three recombinant proteins from *A. fumigatus* [88 kDa, 18 kDa and catalase (17)]. These recombinant proteins were coated (5 μg/mL, 100 μL/well) in 96-well plates overnight at ambient temperature. After washing the wells with phosphate buffered saline (PBS)-Tween 0.05% and blocking with PBS-BSA (1%) for 1 h, sera were added (at 1:500 dilution) to the wells and incubated for 2 h, washes three times with PBS-Tween 0.05%, followed by the addition of horseradish-peroxidase conjugated anti-human IgG (A8667; Sigma-Aldrich) and incubating at ambient temperature for 1 h. After washing three times with PBS-Tween 0.05%, the reaction was developed using *O*-phenylene diamine (OPD), arrested with 4% H_2SO_4 and read at 492 nm.

Statistical Analysis

Performed by one-way variance to compare three groups and Mann-Whitney test for two groups using GraphPad Prism-6.0, GraphPad software (La Jolla California USA).

RESULTS

Description of the Patients

Twelve patients harboring loss-of-function *STAT3*-mutation (STAT3 deficiency) followed at CEREDIH, Necker-Enfants Malades Hospital, Paris, were included in this study. Their age was in the range of 20–55 years, and 67% of them were male. Characteristics of these patients and mutations are detailed in **Table 1**. Neutrophil and monocyte counts were normal in all patients. Immunophenotyping showed lower counts of T cells in one patient, memory B cells in seven patients and NK cells in seven patients (**Table 1**). Among 12 patients, six developed at least one episode of aspergillosis, including aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA) and allergic bronchopulmonary aspergillosis (ABPA) (11). Three *Aspergillus* infections were ongoing at the time of this study. Six-patients displayed heterozygous *STAT3*-R382W mutation. No statistical difference in terms of age, sex, or immunophenotyping was evidenced between the six patients who developed aspergillosis (STAT3-asp) and those who did not (STAT3-w/o-asp).

STAT3-Deficient Patients With Aspergillosis Showed High Amount of Specific Anti-*aspergillus* IgE and IgG

We had access to the sera from 32 STAT3-deficient patients from the French national cohort (including 12 patients in this study). Eleven of them had developed aspergillosis (STAT3-asp: six with chronic pulmonary aspergillosis (CPA), three allergic bronchopulmonary aspergillosis (ABPA), and two had both ABPA-CPA; eight were with ongoing aspergillosis and three in complete remission at the time of our study). We compared them with healthy controls, patients with ABPA or CPA (without STAT3-deficiency) and patients supplemented with intravenous immunoglobulin.

Among the STAT3-deficient patients, all of them ($n = 32$; 100%) showed increased total IgE titers (>114 kU/L) and in 29 patients (91%) specific anti-*Aspergillus* IgE was detected (≥0.1 kUA/L) (**Figures 1A,B**, **Table 2**). STAT3-deficient patients with ongoing aspergillosis had higher serum levels of specific anti-*Aspergillus* IgE titers than STAT3-deficient patients without aspergillosis ($p < 0.01$), whereas no difference in total IgE was observed. Specific IgG against three recombinant proteins of *A. fumigatus* [88, 18 kDa, and catalase] were significantly higher in STAT3-deficient patients with ongoing aspergillosis compared to those without aspergillosis (representative data shown for *A. fumigatus* 88 KDa proteins in **Figure 1C** and **Table 2**; $p < 0.01$). We then used a combination of three criteria including total IgE > 1,000 kU/L (the cut-off used for ABPA diagnosis), specific anti-*Aspergillus* IgE ≥ 0.1 and specific anti-*Aspergillus* IgG (88KDa; titer above the value of 97.5 percentile of the healthy controls was used as cut-off). Five (62.5%) STAT3-deficient patients with ongoing aspergillosis had these three positive tests compared to none in the STAT3-deficient patients without or with prior aspergillosis ($p < 0.01$).

Innate Immune Cells From STAT3-Deficient Patients Display a Normal Clearance of *A. fumigatus* Conidia

Phagocytic and killing capacities of innate immune cells from STAT3-deficient patients were investigated. There were no significant differences in the phagocytosis-killing of *A. fumigatus* conidia by $CD14^+$ monocytes isolated from whole blood samples of STAT3-deficient patients and healthy controls (**Figures 2A–C**). Also, conidial killing by the neutrophils isolated from whole blood samples of STAT3-deficient patients was similar to that of healthy controls (**Figure 2D**). Further categorization of STAT3-deficient patients into those with or without aspergillosis did not show any significant difference in the conidial phagocytosis as well as killing between these two groups, or compared with healthy controls.

STAT3-Deficient Patients Exhibit Low Th17 and IFN-γ Responses to *A. fumigatus*

As no difference was evidenced in the phagocytic function, the secretion of cytokines by peripheral blood mononuclear cells (PBMC) following *A. fumigatus* conidial (swollen-PFA fixed) stimulation was investigated. The production of TNF-α, IL-1β, IL-6, IL-8, and IL-10 by PBMCs in response to one-day interaction with conidia (innate cytokine) was similar in STAT3-deficient patients compared with healthy controls (data not shown). Whereas, after 5 days of PMBCs-conidial interaction, the frequency of IL-17-producing CD4+ T cells (Th17) and the production of TH17 cytokines IL-17 and IL-22 were significantly lower in STAT3-deficient patients compared to healthy controls (**Figure 3**). Also, the frequency of IFN-γ-producing CD4+ T cells (Th1) and the amount of IFN-γ secreted in STAT3-deficient patients upon conidial stimulation of their PBMCS were decreased compared to healthy controls. Among Th2 cytokines, secreted IL-4 was not detected by ELISA

TABLE 1 | Characteristics of STAT3-deficient patients in this study.

Age (years, range)	*STAT3* mutation	Domain of mutation	Aspergillosis (onset)	Current Asp status	Other fungal infection	Antifungal treatment	Other treatment	$CD4^+/\mu l$ (460–1,232)	$CD8^+/\mu l$ (187–844)	$CD19^+/\mu l$ (92–420)	$CD27^+/CD19^+$ % (9–19)	NK/μl (89–362)
30–39	R382W	DNA binding	CCPA (1992) ABPA (2012)	Prior Prior	No	Itraconazole	SC IgG Cloxacillin Aerosol colistine	1,508	624	286	10	104
20–29	R382W	DNA binding	ABPA (2010)	Ongoing	No	L-AmB 2×/week	SC IgG Omalizumab TMP-SMZ, Azithromycin	1,250	600	325	10	175
30–39	R382W	DNA binding	Aspergilloma (1995)	Prior	Cutaneous fusariosis	Posaconazole	IV IgG Cloxacillin	525	360	315	3	45
20–29	R382W	DNA binding	ABPA (2007)	Prior	MCC	Posaconazole	IV IgG TMP-SMZ	760	380	304	3	152
20–29	R382W	DNA binding	No		MCC	Itraconazole	IV IgG TMP-SMZ	924	546	546	5	42
30–39	R382W	DNA binding	No		Cutaneous fusariosis	Itraconazole	TMP-SMZ, Azithromycin	142	191	142	2	42
20–29*	S560del	Linker domain	Aspergilloma (2011)	Ongoing	MCC	Itraconazole	SC IgG	588	384	72	11	84
30–39*	S560del	Linker domain	No		MCC	None	Cloxacillin	891	1269	243	4	189
50–59*	S560del	Linker domain	No		MCC	Fluconazole	None	700	364	140	11	98
20–29	V637M	SH2	ABPA (2005) CCPA (2013)	Prior Ongoing	No	Posaconazole	IV IgG Aerosol AmB TMP-SMZ, Azithromycin	774	396	306	6	72
30–39	I568F	Linker domain	No		MCC	None	IV IgG	918	450	360	9	36
20–29	S668Y	SH2	No		No	Itraconazole	IV IgG Cloxacillin	855	240	225	8	45

**Relatives of the same family. CCPA, chronic cavitary pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis; Asp, aspergillosis; MCC, Mucocutaneous Candidiasis; L-AmB, liposomal amphotericin B; IgG, immunoglobulin G; SC, subcutaneous; IV, intravenous; TMP-SMZ, trimethoprim-sulfamethoxazole.*

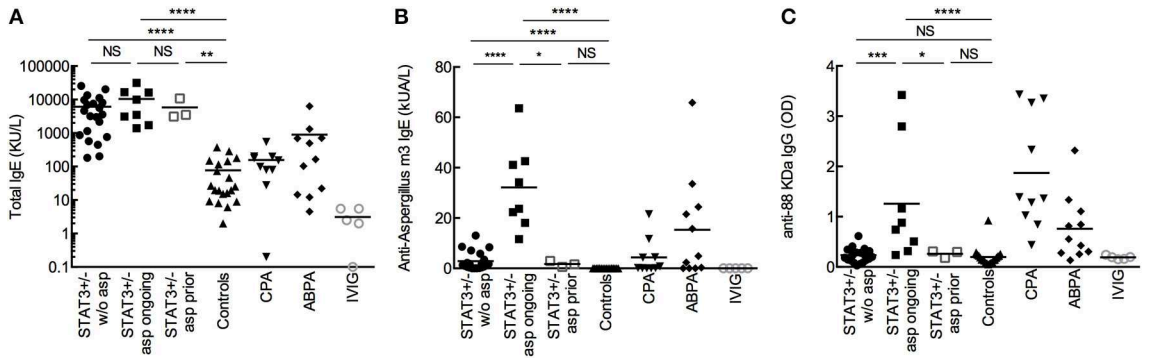

FIGURE 1 | Total IgE **(A)**, specific IgE **(B)**, and IgG **(C)** against *A. fumigatus*. Comparison of sera from STAT3 deficient-patients ($STAT3^{+/-}$, representing heterozygous mutation) without aspergillosis (w/o asp; $n = 21$), with ongoing aspergillosis (asp ongoing; $n = 8$) or with prior aspergillosis (asp prior; $n = 3$), with healthy controls ($n = 20$), CPA ($n = 10$), and ABPA ($n = 11$) patients, and patients receiving substitutive intravenous immunoglobulin (IVIG; $n = 5$). OD: optic density; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ (the mean values are presented in the figures).

TABLE 2 | Total IgE and specific anti-*Aspergillus* IgE and IgG based on aspergillosis phenotype.

Controls/patients	N	Total IgE kU/L Median (range)	Total IgE > 1,000 *N* (%)	Asp-IgE kUA/L Median (range)	Asp-IgE > 0.1 *N* (%)	Asp-IgG 88kDa Optic density Median (range)	Asp-IgG > cut-off* *N* (%)	Triple positive criteria** *N* (%)
STAT3-deficient patients	32	3,527 (182–31,302)	26 (81%)	2.1 (<0.1–63.6)	29 (91%)	0.29 (0.03–3.42)	5 (16%)	5 (16%)
Without aspergillosis	21	3,579 (182–25,472)	15 (71%)	1.3 (<0.1–13)	18 (86%)	0.22 (0.03–0.62)	0	0
With ongoing aspergillosis	8	6,683 (1,397–31,302)	3 (100%)	29.0 (11.6–63.6)	8 (100%)	0.81 (0.24–3.42)	5 (63%)	5 (63%)
CPA	4	10,003 (1,397–31,302)	4 (100%)	33.2 (11.6–63.6)	4 (100%)	0.95 (0.24–3.42)	3 (75%)	3 (75%)
ABPA	2	5,808 (1,721–9,894)	2 (100%)	37.8 (34.2–41.1)	2 (100%)	0.60 (0.31–0.88)	1 (50%)	1 (50%)
CPA and ABPA	2	9,665 (3,108–16,222)	2 (100%)	20.2 (18.1–22.3)	2 (100%)	1.65 (0.51–2.80)	1 (50%)	1 (50%)
With prior aspergillosis	3	3,474 (3,106–10,801)	3 (100%)	1.6 (0.9–2.9)	3 (100%)	0.30 (0.18–0.31)	0	0
Healthy controls	20	22.5 (2–374)	0	<0.1	0	0.15 (0.05–0.92)	1 (5%)	0
CPA	10	125 (0.2–545)	0	0.5 (<0.1–21.5)	8 (80%)	1.38 (0.44–3.43)	9 (90%)	0
ABPA	11	163 (4.5–6311)	2 (18%)	4.9 (<0.1–65.8)	8 (73%)	0.55 (0.13–2.32)	5 (45%)	1 (9%)
Substitutive IVIG	5	2.5 (0–5.5)	0	<0.1	0	0.19 (0.15–0.24)	0	0

*N = number of patients. Asp-IgE, specific anti-Aspergillus IgE; Asp-IgG, specific anti-Aspergillus IgG; CPA, chronic pulmonary aspergillosis; ABPA, allergic broncho-pulmonary aspergillosis; *Specific anti-Aspergillus IgG cut-off defined by the value of 97.5 percentile of the healthy controls. **Triple positive criteria defined by total IgE > 1,000 kU/L, specific anti-Aspergillus IgE > 0.1 kUA/L and specific anti-Aspergillus IgG > cut-off.*

and was lower in STAT3-deficient patients upon FACS analysis (intracellular), whereas IL-5, measured by ELISA, and Treg frequency ($CD4^{+}CD25^{+}Foxp3^{+}$) measured by FACS were not different between STAT3-deficient patients and healthy controls (data not shown).

Low Pro-inflammatory and IFN-γ Responses Are the Characteristic Features of STAT3-Deficient Patients With Aspergillosis

No difference was observed in the innate cytokine levels between STAT3-deficient patients and healthy controls (**Figure 3**). However, when STAT3-deficient patients were categorized with and without aspergillosis (STAT3-asp and STAT3–w/o-asp, respectively), we observed that the secretion of pro-inflammatory cytokines by PBMCs upon interaction with *A. fumigatus* conidia, including TNF-α, IL-1β, and IL-6 (day-1) but also IFN-γ (day-5) were significantly lower in STAT3-asp compared to STAT3–w/o-asp patients (**Figures 4A,B**). In the subgroup of STAT3-deficient patients with aspergillosis, no difference in the IFN-γ, IL-6, TNF-α, and IL-1β secretion were observed among patients with ongoing vs. prior aspergillosis (data not shown). Of note, among STAT3-asp patients, one with ongoing ABPA showed higher secretion of IL-5 and IL-17 and low secretion of IFN-γ (**Figure 4C**).

DISCUSSION

Our study shows no intrinsic innate immune defects, such as phagocytosis and killing, in the patients harboring loss-of-function mutations in the *STAT3* gene (STAT3-deficiency),

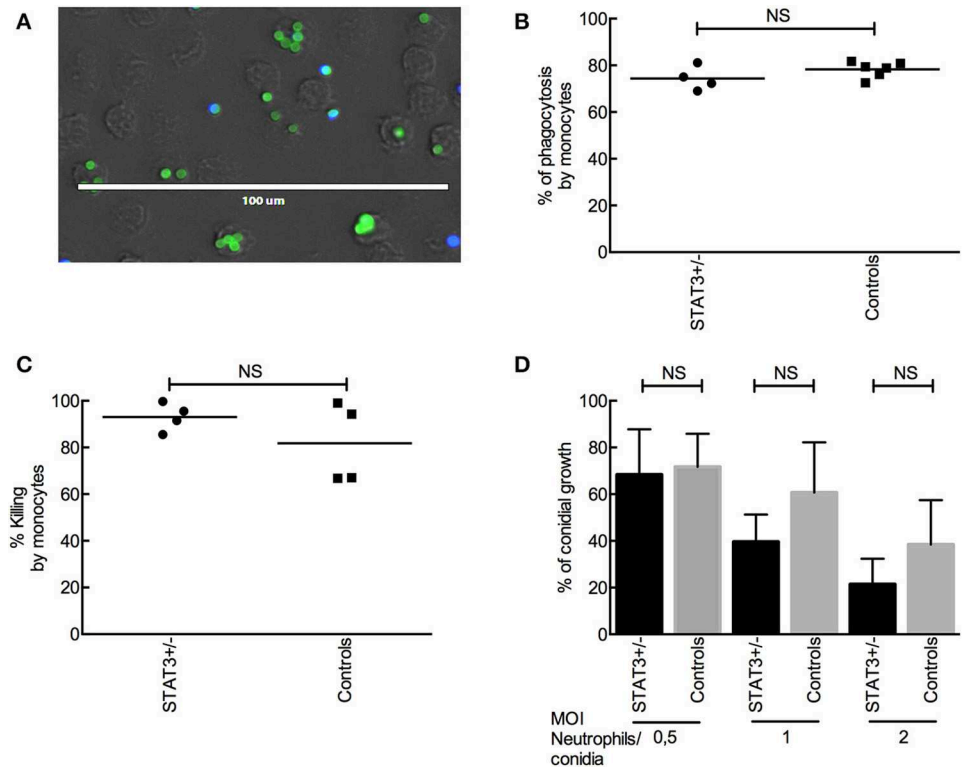

FIGURE 2 | *A. fumigatus* conidial phagocytosis and killing by monocytes and neutrophils. **(A)** Phagocytosis of FITC-labeled conidia (parental strain) by $CD14^+$ monocytes from STAT3-deficient patient ($STAT3^{+/-}$) and controls. Extracellular conidia are labeled with CFW (in blue) whereas intracellular conidia are pre-FITC-labeled (in green). **(B)** Phagocytosis of Δ*ku80* conidia by $CD14^+$ monocytes from four STAT3-deficient patients and six healthy controls. **(C)** Killing of Δ*ku80* conidia by $CD14^+$ monocytes from four each of STAT3-deficient patients and healthy controls, and evaluated by colony forming unit (CFU) counting. **(D)** Killing of Δ*ku80* conidia by neutrophils at different conidia: neutrophils ratios from six each of STAT3-deficient patients and healthy controls.

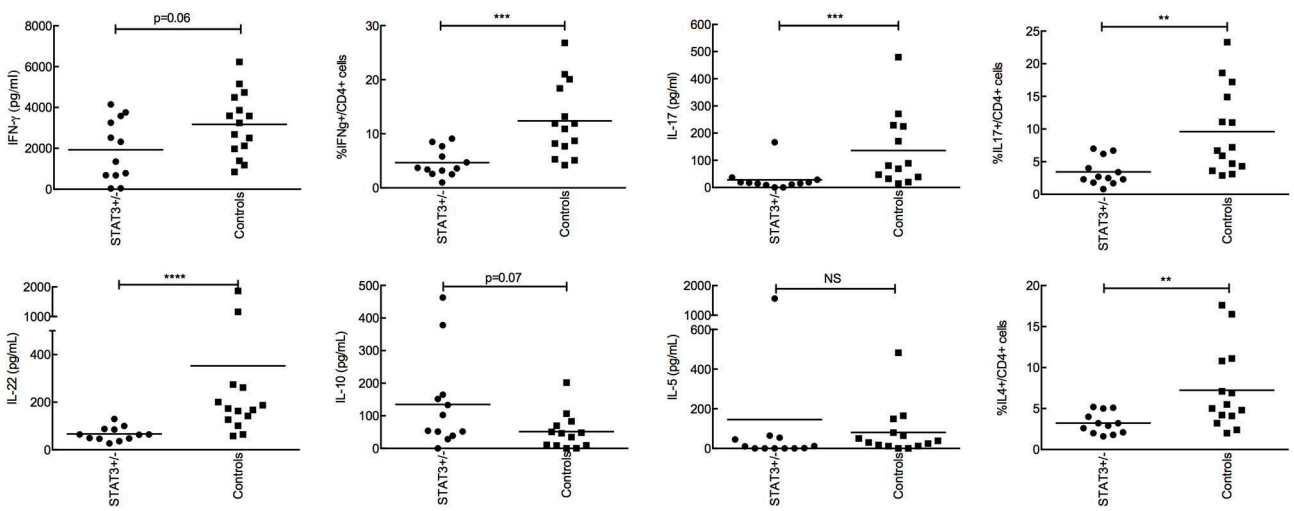

FIGURE 3 | Analysis of adaptive immune response after stimulating PBMCs with conidia for 5-days. 2×10^5 conidia were co-incubated with 2×10^5 PBMC from STAT3-deficient patients ($STAT3^{+/-}$) and healthy controls. Secretion of IFN-γ, IL-17, IL-22, IL-10, IL-5 were analyzed by ELISA and expressed in pg/mL, while, percent CD4+ T cells expressing IFN-γ, IL-17, IL-4 was analyzed by FACS; ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

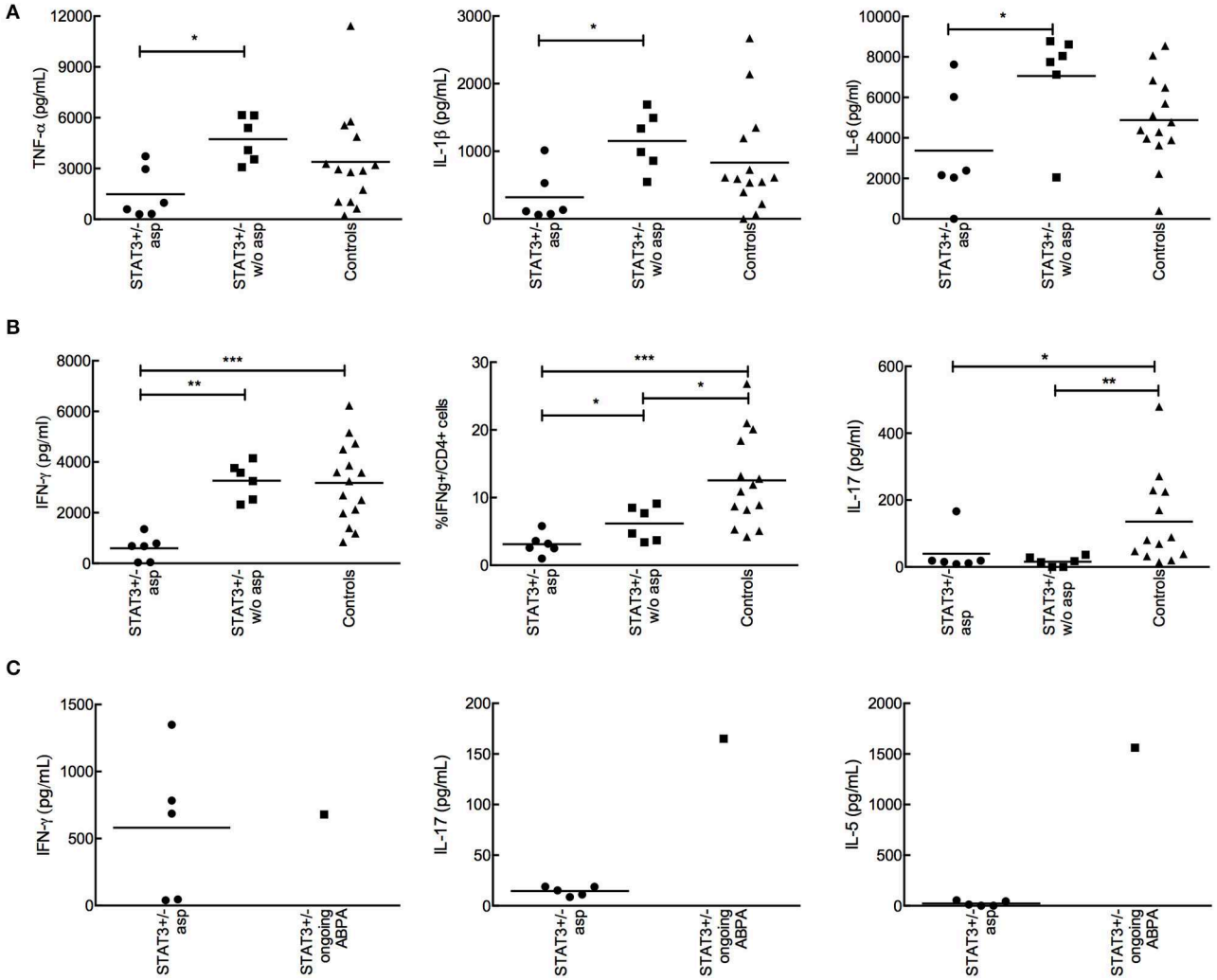

FIGURE 4 | Analysis of the cytokine production by PBMCs isolated from STAT3-deficient patients with or without aspergillosis upon interaction with *A. fumigatus*. 2×10^5 conidia were co-incubated with 2×10^5 PBMC during 1 (innate response) or 5 days (adaptive response). **(A)** TNF-α, IL-1β, and IL-6 secreted by PBMCs isolated from STAT3-deficient patients ($STAT3^{+/-}$) with aspergillosis (STAT3 asp) ($n = 6$), without aspergillosis (STAT3 w/o asp) ($n = 6$) and healthy controls upon one-day interaction with *A. fumigatus* conidia; cytokines were analyzed by ELISA. **(B)** IFN-γ and IL-17 secreted upon stimulation of PBMCs isolated from STAT3-asp, STAT3-w/o-asp and healthy controls with *A. fumigatus* conidia for 5-days; ELISA (secreted) and FACS (intracellular) were performed. **(C)** Analysis of IFN-γ, IL-17 and IL-5 secretion in one STAT3-deficient patient with ongoing ABPA compared to STAT3-deficient patient with aspergillosis ($n = 5$); $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

toward *A. fumigatus*. On the contrary, these STAT3-deficient patients showed a defective adaptive immune response, with lower production of cytokines including IFN-γ, IL-17, and IL-22. Moreover, those STAT3-deficient patients who developed aspergillosis showed further lower level of IFN-γ than the STAT3-deficient patients without aspergillosis. One major protective host mechanism against *A. fumigatus* infection is via Th1 and IFN-γ and a recent study showed that majority of lung-derived T cell phenotype was Th17 upon *A. fumigatus* infection (18, 19). These observations suggest that lower production of Th1 and Th17 cytokines in the STAT3-deficient patients could be the reason for their susceptibility to *A. fumigatus* infection.

STAT3-deficient patients with ongoing aspergillosis had higher specific anti-*Aspergillus* IgE and IgG titers compared to those without aspergillosis. The combination of three positive criteria (total IgE > 1,000 kU/L, specific anti-*Aspergillus* IgE ≥ 0.1 and specific anti-*Aspergillus* IgG above the cut-off) was associated with aspergillosis in the STAT3-deficient population. Further studies are warranted to confirm these results and to identify the adequate cut-off to be used in the diagnosis. We also noticed that substitutive IVIG, often used in STAT3-deficient population, did not falsely increase anti-*Aspergillus* IgG titers in a control population (**Figure 1C**, **Table 2**).

Total and specific anti-*Aspergillus* IgE are used in the diagnosis of allergic forms of aspergillosis (ABPA), notably in those

patients with cystic fibrosis and asthma (20). This was also evidenced in chronic granulomatous disease (CDG) patients (21): elevated total (>1,000 kU/L) and specific anti-*Aspergillus* IgE were noticed in four out of eleven CGD patients. Our group has recently described a CGD patient with *Aspergillus felis* invasive infection who had elevated total and specific anti-*Aspergillus* IgE (22). In another study, a patient with CARD9 deficiency (due to homozygous mutation) and extra-pulmonary aspergillosis had an elevated total IgE level (23). Thus, elevated total (>1,000 kU/L) and specific anti-*Aspergillus* IgE can be observed in the setting of STAT3 deficiency, not only in patients with ABPA-like presentation as evidenced by Duréault et al. (11), but also in other forms of STAT3 deficiency-related pulmonary aspergillosis as evidenced here. In addition, such immunological features can also be observed in other primary immune deficiencies complicated by aspergillosis, including in patients without ABPA criteria. In STAT3-deficient patients an elevated total IgE level is characteristic of the disease, and possibly be explained by IL-21 signaling defect (24). The defective IL-10 (anti-inflammatory) response may lead to an exaggerated immune response resulting in allergic form of aspergillosis (25, 26).

The normal function of neutrophils of STAT3-deficient individuals against *Aspergillus* has already been shown earlier (10, 27); we confirmed these results in our study, and in addition we showed a normal function of monocytes from STAT3-deficient patients for phagocytosis and killing of *A. fumigatus* conidia. Also, innate cytokine production by PBMCs from STAT3-deficient patients was similar to that of healthy controls. Together, this absence of innate immune defect in the STAT3-deficient patients might explain why invasive aspergillosis is very rare in this primary immunodeficiency condition unlike in Chronic Granulomatous Disease (CGD) patients displaying a defective killing of *Aspergillus* by neutrophils, and these patients develop invasive aspergillosis (28, 29). Nevertheless, airway epithelial cells and alveolar macrophages from STAT3-deficient patients have never been studied for *Aspergillus* infection, but we are limited in obtaining these samples.

A defect in IFN-γ response has already been shown in STAT3-deficient patients upon stimulation with heat-killed *Staphylococcus aureus* and *Candida albicans*, two other major pathogens in STAT3-deficient phenotype (9, 30). We also observed a defect in the adaptive IFN-γ secretion from PBMC of STAT3-deficient patients following stimulation with *A. fumigatus*. This lower secretion of IFN-γ by PBMCs was contributed mainly from the STAT3-deficient patients with aspergillosis, and no difference in the IFN-γ secretion was evidenced between the patients with ongoing and prior aspergillosis. The lower secretion of IFN-γ and TNF-α by T cells was also reported upon phytohaemagglutinin (PHA) and anti-CD2/CD3/CD28 microbeads stimulation in AD-HIES patients (31, 32). Some other studies did not demonstrate any defect in IFN-γ production by STAT3-deficient patients (8, 24, 32). This may be explained by different stimuli used (staphylococcal enterotoxin and antigens of *Candida albicans* but not heat-killed pathogens) and by other immune cell studied (neutrophils).

IFN-γ secretion defect after *Aspergillus* stimulation in our cohort is of interest as IFN-γ is the cornerstone in defense against aspergillosis (33). It was shown that in patients with hematopoietic stem cell transplantation and hematological malignancy, an increased IFN-γ response to recombinant proteins of *A. fumigatus* cell wall was associated with improved outcome of invasive aspergillosis (34). Whereas, renal allograft recipients with invasive fungal infection failed to show any increase in the level of IFN-γ (35). Interestingly, mortality due to invasive aspergillosis in experimental murine infection model was related to an impaired IFN-γ response in mice (36), and IFN-γ therapy had a protective role (37). IFN-γ increases killing capacities of human neutrophils and monocytes against *A. fumigatus* hyphae and *A. terreus* and the release of pro-inflammatory cytokines (38, 39). Recombinant IFN-γ therapy was therefore used in several clinical trials to treat or at least to prevent fungal infections (33, 40–42). In a randomized trial with 128-CGD patients, IFN-γ therapy was an effective and well-tolerated treatment that reduced the frequency of serious infections and increased the ability of neutrophils to damage *Aspergillus* hyphae (40, 43). IFN-γ therapy was also able to partially restore immune function in a small open-label series of eight patients with invasive candidiasis and/or aspergillosis (36). HLA-DR expression and secretion of pro-inflammatory cytokines by leukocytes were increased following IFN-γ therapy. Moreover, IFN-γ treatment was efficient to inhibit IgE production in STAT3-deficient patients (44). In the French STAT3-deficient cohort, we identified three patients treated with combination of IFN-γ and antifungals for four episodes of aspergillosis, and two of them showed favorable outcome. Association of IFN-γ with antifungal treatment and surgery limits the specific evaluation of IFN-γ therapy but confirms the urgent need of further studies on IFN-γ therapy in the STAT3-deficient patients with aspergillosis.

We also showed a defect in secretion of IL-17 by PBMC and IL-17-producing $CD4^+$ T cells after *A. fumigatus* stimulation. Defect in IL-17 has already been reported in STAT3-deficient patients after anti-CD3/CD28 monoclonal antibodies, *Candida* or *Staphylococcus* antigen stimulations, leading to a defective production of anti-staphylococcal factors (neutrophil-recruiting chemokines and antimicrobial peptides) by epithelial cells (8, 45). IL-17 defect explain a large part of *Candida* and *Staphylococcus* skin infections in this deficiency and may participate to *Aspergillus* susceptibility.

Of the 12 STAT3-deficient patients included in this study, six of them had mutation in the DNA-binding domain (R382W), four in the linker domain (three S560del and one I568F) and two others showed random mutations in the SH2 domain (V637M and S668Y) in the *STAT3* gene. The comparison of IFN-γ produced according to these mutations indicated that the mutation in DNA-binding domain resulted in a significant decrease in the IFN-γ produced compared to healthy controls ($p < 0.01$) and to patients with a *STAT3* mutation in the linker domain ($p = 0.04$). The difference of IFN-γ produced was not significant between healthy controls and the patients with a mutation in the linker domain or SH2 domain, suggesting

that the type of *STAT3*-mutation, mainly in the DNA-binding domain, may impact on IFN-γ production. No difference was evidenced for other cytokines tested when grouped according to the mutation types.

To conclude, though loss-of-function mutations in *STAT3* gene is a rare primary immunodeficiency, and our study is limited by the number of STAT3-deficient patients, our data indicates that STAT3-deficient patients, particularly those with aspergillosis, exhibit adaptive immune defect by producing lower IFN-γ and Th17 responses toward *A. fumigatus* infection but not any defects in their innate immune functions. The presence of lung cavities in association with defective adaptive immune responses, defective production of antimicrobial peptides and chemokine might partially explain the development of pulmonary aspergillosis during STAT3-deficiency. This warrants innovative immunotherapeutic approaches, based on cytokine, to treat STAT3-deficient patients with severe chronic forms of pulmonary aspergillosis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes Ile de France 2, France, May 4th, 2015. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FD, VA, JB, OL, FL, and J-PL contributed to the conception of the work. All authors contributed to the acquisition, analysis or interpretation of the data, manuscript revision, read, and approved the submitted version. FD wrote the first draft. VA, JB, OL, FL, and J-PL wrote sections of the manuscript. VA and SW edited the manuscript.

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REVIEW
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Environment and Host-Genetic Determinants in Early Development of Allergic Asthma: Contribution of Fungi

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Asthma is a chronic debilitating airway disease affecting millions of people worldwide. Although largely thought to be a disease of the first world, it is now clear that it is on the rise in many middle- and lower-income countries. The disease is complex, and its etiology is poorly understood, which explains failure of most treatment strategies. We know that in children, asthma is closely linked to poor lung function in the first 3-years of life, when the lung is still undergoing post-natal alveolarization phase. Epidemiological studies also suggest that environmental factors around that age do play a critical part in the establishment of early wheezing which persists until adulthood. Some of the factors that contribute to early development of asthma in children in Western world are clear, however, in low- to middle-income countries this is likely to differ significantly. The contribution of fungal species in the development of allergic diseases is known in adults and in experimental models. However, it is unclear whether early exposure during perinatal or post-natal lung development influences a protective or promotes allergic asthma. Host immune cells and responses will play a crucial part in early development of allergic asthma. How immune cells and their receptors may recognize fungi and promote allergic asthma or protect by tolerance among other immune mechanisms is not fully understood in this early lung development stage. The aim of this review is to discuss what fungal species are present during early exposure as well as their contribution to the development of allergic responses. We also discuss how the host has evolved to promote tolerance to limit hyper-responsiveness to innocuous fungi, and how host evasion by fungi during early development consequentially results in allergic diseases.

Keywords: allergy, severe asthma associated with fungal sensitivity, fungi, Low- and lower-middle-income countries, hyperreactivity

INTRODUCTION

Asthma has largely been thought to be a genetic pre-disposition, however, over the last two decades it is becoming more clear that environment may equally play a significant role in the development of the disease (1). Genetic pre-disposition is supported by numerous epidemiological data showing that children born to both parents with asthma or low forced expiratory volume per second (FEV_1) are at higher risk of developing asthma (2). Many studies have also linked specific gene loci or single nucleotide polymorphisms (SNPs) to asthma disease in different parts of the world (3). For example, HLA-DQ, SMAD3, IL-2RB1, IL-33, IL-1RL1, IL-4RA, ADAM33, FOXP3, STAT6, and 17q21 loci, have been linked to asthma diseases in diverse ethnic groups with varying degrees (3–5). What is intriguing about asthma is that it is mainly prevalent amongst urban dwellers compared to rural areas in different regions of the world. Although asthma has always been associated with first world countries, it is clear that it is on the rise in middle to lower income countries and in some of these countries the incidence rates may be as high as Western world countries (6, 7). This points to additional factors that can contribute to the rise in asthma disease, apart from genetic pre-disposition. Indeed, environmental factors are now considered as another possible factor that contributes to the increase in asthma. This stems from many epidemiological data mainly from North America, Scandinavian and Eastern European countries that have demonstrated that children raised in farm environments tend to be protected from asthma, whereas those from urban "cleaner" environments are at higher risks (8–10). A term now widely known as "hygiene hypothesis" coined by David Strachan in the late 80s (10–12). The hygiene hypothesis is primarily based on exposure to microbes or high endotoxin levels in the environment in early life which leads to reduced allergic asthma. Hygiene hypothesis may not fully explain the rise of asthma as some studies have disproved this early exposure to high endotoxins and reduced asthma risks (13, 14). A study by Litonjua et al. (15) in children under the age of 5 years exposed to high concentrations of endotoxin in house dust mites were at high risk of developing wheeze over a 46 months period. Other studies have also found variable influence of endotoxin levels in asthma and atopy development in early life depending on dose, exposure and timing (16). Apart from endotoxin level studies, other studies focusing on Bacilli Calmetté-Guerin vaccination and early *Mycobacterium tuberculosis* infection amongst children in both high- and low-income countries, could not establish an association between infection or immunization with allergen sensitization (17, 18). Other studies have also shown no association between previous infection with *Toxoplasma gondii*, herpes simplex virus, hepatitis A or *Helicobacter pyroli* with allergen sensitization and allergic diseases amongst Spanish and German university students (19). Other factors linked to increase in asthma are the rise of industrialization in many countries which generates large amounts of air pollution. *In utero* pregnant mothers and early life exposure to tobacco smoke is linked to poor lung function and development of chronic obstructive pulmonary disease (COPD) and early mortality (2, 20). Thus, the relationship between early life exposure to microbes or air pollutants and asthma is complex and is likely to be dependent on many factors, such as the nature of exposure, how and when it takes place, host genetic susceptibility and probably many other factors currently not defined (21).

All these do point to the environment being important in asthma pathogenesis. However, other microbes such as fungi have been linked to exacerbations of asthma in both children and adults, suggestive of a non-protective role (22, 23). Naturally, fungi is not detected in high levels in healthy human lung, which had lead researchers to assume that lungs contain no fungus. This is partly due to the fact that inhaled fungal spores are rapidly cleared by both active physical forces such as mucociliary lining the lung epithelium and clearance by innate immune phagocytic cells, such as macrophages. The lack of highly sensitive techniques to detect fungi in sputum or lavage fluid and cumbersome techniques to detect species, partly contributes to low detection of fungi in healthy human lung (24). Nevertheless, some studies have detected certain phyla in healthy respiratory airways including fungi belonging to the genera *Cladosporium*, *Eurotium*, *Penicillium,* and *Aspergillus*. Other genera such as *Candida*, *Malassezia,* and *Pneumocystis* have been detected, but in low abundance (25). It is likely that some of these species of fungi found in lung sampling may also be part of the oral mycobiome, as techniques used to sample lavage fluid or endoscopy can be contaminated with oral cavity fungi. Some fungi are often detected in sputum or lavage fluid in chronic respiratory diseases such as asthma. These fungal species exploit a compromised host defense system, often germinating, and colonizing the airways, causing mycosis which precedes sensitization and exacerbation of asthma symptoms (26, 27). Some of the fungal species associated with asthma are shown in **Table 1**. These include genera *Aspergillus*, *Candida*, *Alternaria*, *Cladosporium,* and *Cryptococcus* spp. amongst others (35, 40). Some of these species isolated from airways or sinusitis of asthmatic patients with TH2-type disease show viability when cultured *in vitro*, showing that infection is essential in disease manifestation (27). Importantly, these patients generally show increased fungal-specific IgE responses, memory phenotype when stimulated with fungal antigens and eosinophilia which is directly involved in fungal killing (28, 41). It is now clear that fungal colonization contributes largely to asthma and its severity, however, current methods used to determine colonization rely on culture methods for viable fungi and molecular methods which do not necessarily differentiate between active colonization and fungal fragments. This leads to underestimation of disease burden and bias in distinction between disease and colonization depending on detection methodology used. The mode of sensitization is mainly aerosol exposure, however, some fungal species are commonly found colonizing the skin, for example *Malassezia* spp. and *Trichophyton* spp. This suggests that there might have a different mode of sensitization when found on the skin which leads to asthma. These fungal species, although generally found colonizing the skin have also been detected in the both healthy and asthmatic lung and gut which may suggests diverse adaptation in multiple barrier sites (35). In this review, we mainly focus on how some fungi may cause or contribute

TABLE 1 | Some of the fungal species associated with allergic asthma via various routes of sensitization.

Fungal genera	Fungal species	Allergens	Activity	Route of exposure	Reference
Aspergillus spp.	*A. fumigatus* *A. nudilans* *A. versicolor* *A. flavus*	Asp f 1 Asp f 3 Asp f 11, Asp f 27 Asp f 22	Ribotoxin Perioxisomal membrane protein Protein folding Enolase	Inhalation	(27–29)
Alternaria spp.	*A. alternata*	Alta 1 Alta 2 Alta 4	Transporter, disarms defense Protein initiation factor Thioredoxin	Inhalation	(22, 24, 30, 31)
Cladosporium spp.	*C. clasosporioides* *C. herbarum*	Clac c9 Clac c14 Clac h8	Peroxysomal protein Transaldolase Mannitol dehydrogenase	Inhalation	(32–34)
Malassezia spp.	*M. globosa* *M. furfur* *M. sympodialis* *M. restricta* *M. japonica*	MGL_1304 Mala f 2-4 Mala s 1-11, MalaEX	Not known Stress responses Protease activity Vesicle trafficking and innate immune responses	Skin contact Inhalation Ingestion	(35, 36)
Cryptococcus spp.	*C. neoformans* *C. gattii*	Chitin Chitosan Mpr1	Immune activation CNS invasion	Inhalation	(37, 38)
Trichophyton spp.	*T. interdigitale* *T. tonsurans* *T. mentagrophytes*	Trit 1 Trit 4	Enzymatic activity? Sporulation?	Skin contact	(39)

to allergic asthma, we also discuss how fungi such as *Malassezia* and *Trichophyton* that mainly colonize the skin can be important sources of allergens that sensitizes the skin and cause allergic asthma through atopic march (42). We discuss some of the genetic risk factors associated with fungal asthma and tolerance mechanism that host has developed to limit hyperreactivity to these ubiquitous organisms.

FUNGAL SENSITIZATION AND ASTHMA RISKS

Sensitization to fungi poses a major risk factor for asthma including severe form of asthma and death. Sensitization to fungi is estimated anywhere between 2 and 90% in asthmatics (24, 43). Factors that affects this variation include different exposure, batch of commercially available skin prick test (SPT) extracts and subjective methods used to measure exposure. There is also a high degree of multiple fungal species sensitization, which further complicates these estimates. For example a study done in multiple states within the United States of America using a Pharmacia Capsulated hydrophobic carrier polymer (CAP) system, did show a positive correlation between sensitization to outdoor *Alternaria* and indoor *Cladosporium* (44). Four fungal genera namely, *Alternaria*, *Aspergillus*, *Penicillium,* and *Cladosporium* are overly represented when it comes to measuring fungal allergen exposure (43, 45, 46). This is partly due to airborne abundance, distinct morphological characteristics, geographic locations, and whether indoor or outdoor allergens are being considered.

Highest asthma exacerbations are observed during sporulation seasons, however, most individuals are exposed to either sub-micron ($>1\,\mu m$) or larger ($<1\,\mu m$) fungal fragments (23, 43, 47). These fungal fragments are derived from broken or fractured conidia or hyphae and can be easily dislodged by penetrating deeper pockets of the lung at much higher concentrations than spores or conidia (46, 48, 49). This is particularly an efficient aerosolization model for some of the larger fungi such as *Alternaria alternata* that can be over $10\,\mu m$ in size and can only penetrate upper respiratory airways (48, 50). Determining whether an individual patient has been sensitized through fungal fragments or live infection is difficult to ascertain without a positive culture, or biopsy for histopathology. However, several case reports have shown that fungal mucosal infection precludes sensitization to that fungus and exacerbates life-threatening allergic asthma (27, 51, 52). Treatment with antifungal drugs and low dose corticosteroids improves asthma symptoms and clears fungal infection, suggesting that airway mycosis may be important in subsequent sensitization (23, 51, 53). It is likely that infectious mycosis instigates sensitization through activation of TH2-type immune response, while fungal fragments derived from hyphae and conidia can exacerbate allergic asthma through their easily accessible fungal pathogen associated molecular patterns (PAMPs) (46, 49). Several animal model studies also support the idea that airway mycosis induces TH2-type airway disease and that some fungal PAMPs may exacerbate allergic asthma in the presence of allergens such as ovalbumin or house dust mite (28, 54–56).

Some studies have suggested that infants are likely to be exposed more than adults and more fungal fragments seem to seed deeper pockets of the lung, which may explain increased risk to fungal induced asthma (49, 57). However, variation in exposure and allergic asthma has also been observed in children from low-income and high-income homes, which may

suggests that exposure is also biased based how often one visits a physician. Children from high income homes are likely to be diagnosed with fungal induced asthma simple because of access to healthcare, whereas children from low income homes may even have higher exposure due to living in damp, moldy homes, with less access to healthcare and diagnosis (58).

GENETIC BASIS FOR FUNGAL INDUCED ASTHMA

Asthma is a multifactorial complex disease whose origins and pathogenesis are still not clear. Multiple genetic risk factors involved in inflammatory pathway of allergic asthma have been reported. These polymorphisms associated with asthma risk are not present in all patients and seem to be influenced largely by environment. Interleukin 4 receptor alpha (IL-4Rα) and IL-13 polymorphisms are associated with hyper IgE and asthma severity (59, 60). There are currently eight known naturally occurring SNPs of the IL-4RA namely ile75val, glu400ala, cys431arg, ser436leu, ser503pro, gln576arg, ser752ala, and ser786pro (61, 62). Some of these SNPs are associated with *A. alternata* moderate to severe asthma in children (63). HLA-DQB1*03 and HLA-DRB1*13 have also been associated with *A. alternata* moderate to severe asthma in children, where HLA-DQB1*03 was reduced in frequency and HLA-DRB1*13 increased in frequency when compared to *A. alternata* mild asthmatic children (63). These SNPs were specific to fungal sensitization as they were not present in control Allergic Bronchopulmonary Aspergillosis (ABPA) or Chronic Pulmonary Aspergillosis (CPA). Other studies have shown susceptibility variants specific to innate immune responses and pattern recognition. Toll-like receptor 3(TLR-3), TLR-9, DECTIN-1 variants were shown to be associated with severe asthma with fungal sensitization (SAFS) induced by *Aspergillus fumigatus* in a Caucasian older cohort (64, 65). In another study, DECTIN-1 variant rs58677678 was found to be associated with reduced gene expression leading to unrestrained type 2 immune response and subsequently poor lung function (66). Other SNPs associated with SAFS in this cohort were genes associated with TH2 chemoattractant (CCL17) and monocyte recruitment (CCL2) or immune suppression (IL-10). Interestingly, even in this cohort, there were no correlation in the SNPs between SAFS, atopic asthma and healthy controls (64). These SNPs were specific to SAFS and there were major genetic differences even between closely related ABPA disease (64, 65). To fully appreciate the function of these variants in susceptibility to disease, it would be of great benefit to combine experimental models to delineate function of each variant in that gene. This was perfectly illustrated in patients with invasive Aspergillosis where variant rs35699176 in ZNF77 was shown to cause loss of integrity of the bronchial epithelium and allowed fungal growth (67). This variant was recapitulated *in vitro* by genetically-editing immortalized epithelial cells via CRISPR/cas9 which were subsequently infected with *A. fumigatus* conidia. Such studies in SAFS could lead to better understanding of gene function in disease susceptibility, especially in cases where human tissue is not easily accessible. It is worth noting that variants in the study by Knutsen et al. (63) were from 10 year old asthmatic children from multi-ethnic groups. This is relevant in Africa, where there are much larger genetic variations expected compared to Caucasian ancestry.

Several of the genetic polymorphisms associated with fungal allergic asthma are in the innate arm of the immune response to fungi, particularly pattern recognition receptors (PRRs) that recognize evolutionary conserved PAMPs. Although a full discussion is beyond this review, as we have recently reviewed this topic in great length (68), it is worth highlighting PRRs that are implicated in fungal induced asthma. Fungi is covered by cell wall which is mainly composed of outer mannan layer, a β-glucan layer and an inner chitin layer. β-glucan is a sugar found in most fungi and varies in content depending on the fungal species and stage of germination. Beta-glucan is recognized by Dectin-1 mainly by phagocytes and upon recognition initiates a cascade of events including phagocytosis, NF-κB activation, induction of pro-inflammatory genes including GM-CSF, TNF-α and activation of TH2 cytokine secreting cells. Studies in both human and mouse are contradictory regarding the role of Dectin-1 in the induction of TH2 cytokines (68). Several human studies support the idea that Dectin-1 engagement with its ligand favors TH2 environment either through instruction of naïve T cells to secrete TH2-type cytokines (69) or releasing of chemokines (CCL20) associated with TH2 cell recruitment (70). However, other studies suggest that Dectin-1 inhibits TH2 polarization through favoring a TH17 cell polarization or direct inhibition of IL-33 secretion (66, 69). In animal models, the role of Dectin-1 in allergic asthma is even more complicated, with several independent studies showing considerable inconsistences (54, 55, 71–76). Beta-glucan availability on the surface *Aspergillus versicolor* or *Cladosporium cladosporioides* live conidia determines the importance of Dectin-1 in TH2 airway immune responses (74). So, future studies should ascertain how each DECTIN-1 variant directly contributes to fungal induced asthma using both purified ligands and multiple fungal species with varying morphologies and growth stages as all these factors are likely to be important.

Toll-like receptors are another group of PRRs that have been shown to bind to fungi and induce pro-inflammatory cytokines (77–79). Several TLRs have been shown to recognize various PAMPs on the surface or released nucleic acid of fungi for example TLR2, TLR3, TLR4, TLR6, and TLR9 promote inflammation and clearance (80). TLRs can bind to fungal PAMPs individually or as heterodimeric clusters and have also been reported to cooperate with CLRs to induce immune responses (77, 81, 82). TLR3 SNP (rs10025405) rare G allele genotype results in susceptibility to SAFS. TLR3 is involved in direct recognition of *A. fumigatus* and upon recognition by epithelial cells activates a TRIF pathway leading to indoleamine 2,3-dioxygenase activation and cytokine production. Mice deficient of TLR-3 are susceptible to pulmonary *A. fumigatus* infection which may explain this susceptibility genotype (83). Currently, mechanisms of how TLR-3 or TLR3 SNP (rs100254050) can lead to susceptibility to severe asthma are unclear. TLR9 is an endosomal TLRs whose expression has been shown to be upregulated by Dectin-1 upon stimulation with *A. fumigatus*

conidia or hyphae. TLR-9 regulates airway hyper-responsiveness and inflammation when sensitized and challenged with resting *A. fumigatus* conidia. Single nucleotide polymorphism in TLR-9 (rs352140) specifically the rare CC and CT genotypes are associated with SAFS (64). This suggests that TLR-9 plays an essential part in the modulating *A. fumigatus* induced allergic asthma. Mechanistically, it is likely that TLR-9 regulates allergic asthma induced by resisting *A. fumigatus* through regulating IL-5, IL-13, CCL11, and CCL21 and this process seems to be independent of Dectin-1 (84). During swollen *A. fumigatus* induced allergic asthma, TLR-9-deficient mice are more susceptible and show increased fungal growth. In this setting, TLR-9 completely regulates dectin-1 expression and by extension IL-17A expression, which explains increased fungal burden in the absence of TLR-9 (84).

Large birth cohort studies that primarily focus on the genetic-environmental interactions in the first year of life with follow-up of these infants up to at least 10 years of age can give greater insights into asthma development. Most risk genetic studies have ignored this crucial early phase where environmental exposure exerts most of its influence in the development of asthma. The importance of these studies was demonstrated in a 17q21 locus risk alleles ORMDL3 and GSDMB, where the same genotype associated with risk was influenced by environment to be protective (21). It would be of great interest to evaluate some of the fungal asthma associated risk alleles in birth cohort studies where environmental exposure at infancy if taken into account.

HOW IS ALLERGY TOLERANCE GENERATED TO FUNGAL SPECIES?

Fungi is ubiquitous in our environment and it is estimated that in some seasons an individual can be exposed to as high as 50,000 spores per day, yet most individuals do not react and remain non-sensitized (49). Development of tolerance to fungal allergens is intricately regulated by a balance between regulatory T cells (Tregs) which suppress exaggerated inflammation and effector TH2 cells, which are essential in preventing invasive airway mycosis (85). Allergy is usually associated with loss of tolerance to highly allergen-specific TH2 cells and B cells producing IgE (86). In fungal induced TH2-type allergic airway responses, loss of tolerance to fungal allergens can be viewed as an active process aimed at limiting suppressive immune responses in favor of a robust TH2 airway inflammation capable of containing invasive and dissemination of infection to other organs such as the brain (27). How the immune system choses to react to some innocuous antigens or maintain tolerance to majority of inhaled airborne particles is poorly understood. This is particularly of great interest in fungal induced asthma where both active TH2 immunity and immunoregulation are required for initial host protection against invasive fungal species and limiting detrimental pathology. In fact most allergic individuals are extremely tolerant to majority of airborne allergens encountered, except for few that cause aberrant allergic disease (85, 87). Studies specifically focusing on T reg function in allergic neonates suggests an early dysfunction of these cells due to reduced expression CTLA4, TGF-β, resulting in failure in restraining a skewed thymic and peripheral TH2 cells (88). This is also supported by primary immune-deficiency studies in atopic neonates with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome where FOXP3 gene is impaired. These patients present with multiple organ autoimmunity and neonatal development of allergies due to unrestrained TH2 responses at mucosal surfaces (89). There are various mechanisms in which T reg cells can induce immune tolerance to allergens. These mechanisms do include active tolerance where both thymic or peripheral T regs actively suppress allergen specific naïve T cells, allergen ignorance and immunosuppression through secretion of IL-10 by Type 1 T regs (Tr1) (86, 89). The latter is not induced *de novo* and only seem to play a role in an already developed TH2 allergic disease, which makes it less attractive as therapeutic intervention in most allergic disease, but may well be beneficial in fungal induced asthma, which needs a balanced TH2 immunity and reduced host pathology (86). Two mechanisms seem to be relevant to fungal tolerance, namely direct suppression of allergen-specific TH2 cells by allergen specific T regs and allergen ignorance (85). The former is supported by studies using antigen reactive T cell-enrichment (ARTE) assays in sensitized individuals, where *A. fumigatus*-specific Treg cells directly compete with *A. fumigatus*-specific TH2 cells (85). Interestingly, this allergen suppression seems to be specific to particulate *A. fumigatus* allergens and rare in soluble allergens (85, 90). Allergen ignorance mechanisms of tolerance does suggest that most major fungal allergens are ignored by the immune system of healthy individuals. Studies using MHC multimer enrichment showed that allergen-specific T cells from healthy individuals were neither clonally expanding nor proliferating when exposed to antigen *in vitro* or during allergen season and resembled a naïve phenotype when compared to asthmatic individuals (91). Currently, it is unclear whether only certain fungal allergens are specific for inducing T regs or all allergens have a potential to induce both TH2 cells and T regs depending on the local cytokine milieu or antigen load. It is likely that pre-disposed individuals without disease possess a reasonable Treg compartment which keeps all airborne allergens at bay. However, during a low-grade fungal infection, this stable T reg compartment is highjacked leading to TH2 allergic immune response which keeps fungal load low and prevent dissemination (27, 85). This idea is supported by studies where chronic exposure to low grade *A. niger* is able to break tolerance induced by inert ovalbumin antigen in favor of a TH2 immune response (56). It is likely that there are numerous ways in which the human host has co-evolved with fungal airborne species and developed an intricately balanced tolerance vs. necessary inflammation to keep the pathogens out. It is likely that these tolerance mechanisms develop from birth and are nurtured throughout life, acting alone or in synergy with others to limit immunopathology. How tolerance induced by T reg cells toward common ubiquitous fungal airborne allergens is abrogated is of significant interest in the field of fungal induced asthma. Understanding mechanisms of tolerance to airborne fungi initiated by both neonatal and adult human thymic and peripheral Tregs would lead to not only new therapeutics that

prevent this break of tolerance, but also interventions that limit immunopathology without compromising active fungal clearance. Appropriate mouse models that closely mimic the spectrum of fungal induced allergic asthma need to be developed in order to delineate mechanisms of tolerance.

FUNGAL ASTHMA IN EARLY-LIFE, HOW CAN WE UNDERSTAND IT?

Asthma is thought to be a disease that develops very early in life with almost a third of children under 5 years showing signs of wheezing. Lower respiratory infections contribute to poor lung function in infants who develop early onset of the disease. This early poor lung function is thought to persist until adulthood and is associated with premature mortality (20). Exposure to fungi in the first 2–3 months of life is generally associated with exacerbations of asthma in children (9, 22, 23, 30, 92). However, other studies in large birth cohorts that were followed from birth until age 13 have suggested that indoor fungal sensitization by certain dustborne yeasts were associated with protection (93). The mechanisms of protection to dustborne yeasts are unclear, but it is possible that because of hand-to-mouth feeding habits in infancy, these infants develop tolerance to such airborne yeast due to gut-specific immune responses that favor tolerance toward same ingested yeast (93). Other mechanisms of protection could be due to differences in species on dustborne vs. airborne fungal species which may also result in differential PAMPs exposure and subsequent stimulation of protective PRRs such as TLRs or C-type lectin receptors.

Lung is incompletely developed at birth and completes its post-natal development in the first 3 years of life (94, 95). This post-natal development is largely shaped by encountered external environment and means that the lung remains vulnerable to pathogen colonization during these first 3 years of life (96, 97). What is interesting about these early stages of life, is that pregnant mothers show a TH2 biased immune responses which is transferred to off springs who are also dominated by this skewed TH2 immunity (88). This inherited allergic status of infants from mothers is well-documented and is influenced by other factors including early exposure to microbes, cesarean delivery, breastfeeding, use of probiotics to name a few (96, 98). This TH2 skewed immune response reduces gradually during the first 2 years of life, however, allergic infants have been observed to retain this *utero* TH2 bias, which is thought to increase wheezing at this stage (99).

The data regarding immunological changes in the airways in early life is scarce and this is mainly due to invasive procedures required to get BAL samples or histopathology samples. This has also made it difficult to match clinical expression of asthma with changes in immune cells. The evidence which suggested both lung histopathological and physiological changes in children before age 3 who went on to develop asthma before pre-school gave some insights on early respiratory symptoms (100). Histopathological features of asthma have been observed after 3 years of age and include airway smooth muscle remodeling, variable reticular basement membrane and eosinophilic infiltration, a feature not seen in wheezing infants under 2 years old (100, 101). The lack of proper bronchoscopy control subjects in these studies makes it difficult to draw conclusions as it is unclear if this remodeling is due to the atopic disease or normal development of the lung. What also complicates these observations is that these infants are usually suspected of having severe respiratory symptoms and would be under corticosteroids, which may supress inflammatory cells such as eosinophils (96, 98).

To fully appreciate early determinants of asthma pathophysiology several studies have recapitulated these early events in animal models of allergic asthma (102–106). At steady state and during HDM exposure, innate lymphoid cells (ILC2s) producing IL-13 dominate the first 10 days of life in an ST2-dependent manner. This creates a TH2-biased immune phenotype, characterized by M2 macrophages that favor resolution of inflammation and protect the vulnerable developing lung (92, 102, 106). ILC2 have also been detected in sputum of children with severe form of asthma not responsive to corticosteroids, which suggests that these cells are essential in the early years in wheezing (107).

In humans, most studies in infants have mainly focussed on how certain bacterial taxa and viruses influence susceptibility to asthma in the first 3 years of life (96, 98). The information on fungal influence in the development of asthma at early stages is scarce and has only been looked at in the context of gut microbiome, where abundance of *Candida* and *Rhodotorula* species were associated with wheezing in 12 month old birth cohort (108). There are limited neonatal mouse studies focusing on how fungal species exacerbate allergic asthma. Those limited studies have solely focused on *A. alternata*, which is mainly associated with severe type of asthma, a disease not only restricted to children, but found in adults too. These studies have shown that *A. alternata* induces IL-33 dependent steroid resistant asthma (30, 105, 109, 110), which closely resemble that in children non-responsive to oral corticosteroids. This field is still in its infancy and more work is needed to combine asthma clinical data in pediatrics, fungal triggers in those environments and experimental neonatal immunology to decipher context specific disease mechanisms. Considering how little information is available in Africa on fungal sensitization and fungal species associated with different types of asthma, it is conceivable that disease mechanisms will differ, uncovering context specific drivers of allergic diseases. Below, we discuss some of the fungal species that have been associated with asthma.

ALTERNARIA ALTERNATA IN ASTHMA

Alternaria sp. belongs to Ascomycota phylum which is the second most abundant fungal species. It is a cosmopolitan organism that is often isolated with outdoor environments, easily growing on dead vegetation or soil samples particularly during cultivation season of grainy plants (111). Alternaria can be found in rural and urban places particularly in warm climates and at higher CO_2 levels, conditions that favor sporulation and high antigen content per spore (111). Spores have also been found in indoor environments in places such as basements or evaporative coolers (air-conditioning) where it is thought to be an important source of spores that induce exacerbations (48). Although sensitization

to most fungal species is associated with asthma exacerbations, sensitization to *A. alternata* appears to increase the risk of potentially fatal asthma leading to higher hospital admissions under intensive care units (22, 45, 112). In a 25 year birth cohort prospective study done in the US, sensitization to *A. alternata* at age 6 was associated with increased hyperresponsiveness from ages 11 to 26 years, even in children who did not show signs of asthma (113). In another prospective study in Western Australia in 9-year-old children, *A. alternata* sensitization was found to strongly correlate with poor lung function when compared to sensitization to other allergens. Increases in AHR was associated with increase in *A. alternata* spores during dry season with spore counts reaching 300 spores/m^3/day over 1 month (114). In the UK, Central and Eastern Europe, asthma exacerbations, high oral steroid usage and hospitalization have been linked with high *A. alternata* spore content and high allergen load (30, 31). Other retrospective studies have found as high as 20% *A. alternata* sensitization amongst mid-year school children from different locations in the US using more sensitive tests such as CAP system (44).

Most of what we know in terms of immune responses to *A. alternata* has mainly come from mouse studies, where TH2 cells have been demonstrated to play a superior role in the pathogenesis (30, 110, 115–117). In adult asthmatics or children directly sensitized or acutely exposed to *A. alternata*, immune responses have often not been done. In one pediatric study of children under 16 years, who were sensitized to *A. alternata* and had SAFS and unresponsive to oral steroids, they were found to have increased levels of IL-33 (30, 109). The mechanisms of steroid resistance induced by *A. alternata* were further evaluated in a neonatal mouse model where mice were chronically exposed to *A. alternata* and treated with steroids. IL-33 was found to be essential in *A. alternata* induced steroid resistant asthma by promoting TH2 cells and ILC2 that were not inhibited by steroids (30). Other studies have also shown a critical part of TH2 cells, ILC2, signaling molecule STAT6 and ST2 receptor in *A. alternata* induced allergic asthma (110, 115, 116, 118). Although these studies shed some light on immune mechanisms that lead to steroid resistance, it is currently unclear what factors within *A. alternata* organism leads to severe forms and fatal asthma. This is further complicated by lack of understanding on the function of major allergens, Alta 1 and 2. Serine proteases released by *A. alternata* have been shown to cleave IL-33, therefore leading to TH2 allergic airway responses (30, 110). More studies are needed from different geographic locations, socioeconomic status and age groups to elucidate the link between early sensitization to *A. alternata* exposure and development of steroid resistant asthma (44, 113). The function of innate immune responses and PRRs is less studied in both animal models and human challenge models, which can be explained by lack of studies looking at the cell wall properties of this environmentally important fungus.

ASPERGILLUS SPECIES IN ASTHMA

Up to 50% of asthmatics are also sensitized to fungi, presenting with increased IgE specific to fungi. The main culprit fungal species that are often found colonizing the lower airways of the asthmatic lung are *A. fumigatus* and other thermoresistant *Aspergillus* genera species (43). *A. fumigatus* is ubiquitous and is also found in airways of healthy people, where it does not cause any adverse effects and cleared. In *A. fumigatus* sensitized asthmatics, this fungus is associated with severe asthma which is characterized by frequent bronchiectasis, poor lung function and poor control of asthma. Sensitization to fungi, particularly *Aspergillus* with severe asthma symptoms has been classified as SAFS (43, 45, 119). Although SAFS is commonly used, however, this term is restricted to adult asthma and moderately used in children, partly because sensitization in children is often associated with immature immune system and difficulty in diagnoses. So, the term that has been adopted is fungal asthma (120).

Aspergillus-associated asthma is often masked or misdiagnosed with other *Aspergillus* associated diseases such as ABPA which in itself can be worsened by CPA (43, 119, 121, 122). Often, *Aspergillus* sensitized asthmatics do not meet the criteria for ABPA as their total IgE levels fall well-below threshold of (>410 IU/L or 1,000 ng/mL or >1,000 IU/L) and often present with normal levels of IgG (47, 121, 122). The other defining factors are lack of frequent pulmonary infections or bronchiectasis and fleeting shadows often seen in ABPA patients. CPA differs significantly from allergic asthma as it presents with morbidity associated features such as weight loss, productive cough, shortness of breath, pulmonary/pleural cavitation, localized fibrosis, and sometimes aspergilloma (47, 123). A few randomized clinical trials have shown a beneficial effect of antifungal drug (itraconazole) in more than 60% of patients with severe asthma sensitized to fungi, demonstrated by reduced IgE, exacerbations, and improved lung function and quality of life (124). Vericonazole, another antifungal drug have been shown to be effective against CPA (123), but had no effect in a randomized trial of moderate to severe asthmatics sensitized to *A. fumigatus* (125). Discontinuation of antifungal therapy is usually associated with rebound of asthma symptoms in both children and adults (124, 126), which highlights the difficulties in controlling environmental exposure to fungal allergens. Corticosteroids are commonly used to alleviate allergic asthma symptoms in SAFS, however they also pose a risk in developing invasive fungal dissemination (40). Immune responses to *A. fumigatus* associated sensitization are well-described in both animal models (28, 56) and human studies (127).

A. fumigatus lung infection is associated with TH2 cells, cytokines IL-4, IL-5, and IL-13, eosinophils and AHR. Severe form of the diseases is associated with TH17 cells, IL-17A and neutrophilic inflammation (128–130). However, some severe forms of the diseases are associated with hyper IgE and eosinophilia and resistance to corticosteroids (54). Recently, it was shown that there is a considerable cross-reactivity between *Candida albican*-specific TH17 cells residing in the gut and the vast majority of lung associated aero-allergens from *Aspergillus* spp. (127). These *C. albican*-specific T cells broadly promoted pathogenic TH17 cells against *A. fumigatus* in the lung and were associated with immunopathology. This gut/lung cross-talk has been observed in experimental mouse models where gut axis

dysbiosis due to the use of antifungal drugs exacerbates allergic asthma (131, 132). What is characteristic of this lung responses is the proliferation of lung resident *A. fumigatus* and other aero-allergens as *C. albicans* disappear in the gut (131).

CLADOSPORIUM CLADOSPORIODES IN ASTHMA

Cladosporium spp. are dematiaceous fungi and are found in a wide variety of habits, mainly foods. *Cladosporium* spp. are prevalent in indoor environments including moldy homes and are associated with severe forms of asthma particularly during sporulating season (133). *Cladosporium* spp. have been isolated in people's homes and air conditioners at work places and in some cases chronic exposure lead to hypersensitivity pneumonitis, hemorrhagic pneumonia, pulmonary fungal balls, left bronchial obstruction and lesions (32, 33, 134, 135). In some cases, *Cladosporium* spp. were associated with death, although it is unlikely that it was the sole cause of death, as some of the patients had underlying conditions such as diabetes milieus or cavitary lungs (32, 135). *Cladosporium cladosporiodes* is the major species detected in most atopic asthmatics followed by *Cladosporium herbarum*. Some studies have estimated that as high as 38% of atopic asthmatics are sensitized to *C. cladosporiodes*, which is in a similar range to other aero-allergens such as *Aspergillus* (136). This is based on immunoblot serum reactivity to major allergens such as serine protease (Cla c14) or peroxisomal protein (Clac c9) (**Table 1**). It is worth mentioning that there is a certain degree of overlap in amino acid sequence and IgE cross-reactivity between *C. cladosporiodes* and *Penicillium* spp., which can complicate diagnosis as both these species have a similar habitat and modes of human exposure (34). The mechanisms of how *Cladosporium* spp. or its individual components cause or exacerbate asthma are scarce and require further research. Some studies using secreted proteases suggest that proteases can activate allergic airway immune responses by cleaving key components of the lung epithelial barrier such as occludins resulting in a leaky barrier and more access for other allergens which leads to a co-ordinated local inflammatory cascade (137). It is also likely that *Cladosporium* spp. proteases act in a similar fashion to other fungal proteases by degrading specific endogenous substrates of which their products can bind directly to TLR-4 or to protease activated receptor 2 which then activate downstream signals through their cytoplasmic tails leading to recruitment of eosinophils, innate type 2 lymphoid cells and systemic IgE release (137–140). The importance of *C. cladosporiodes* proteases in the induction of allergic airway responses has been demonstrated in mouse models of allergic asthma (74, 75). In these models, chronic exposure of mice to live conidia induced a strong TH2 allergic airway disease characterized by eosinophilia, AHR, elevated levels of IgE and mucus production. However, when *C. cladosporiodes* conidia is inactivated by heat killing, it induced less AHR and allergic responses due to increased exposure of fungal β-glucan and Dectin-1 dependent neutrophilic and TH17 responses (74). These studies suggested that the factor that promotes TH2 allergic asthma in *C. cladosporiodes* where less β-glucan is exposed is likely to be proteases, although this was not formally investigated in this study.

CRYPTOCOCCUS NEOFORMANS IN ASTHMA

Cryptococcosis is largely associated with immunocompromised patients and is the most common cause of HIV-related meningitis particularly in low CD4 count, killing over 150,000 people in Sub-Saharan Africa (141, 142). *Cryptococcus neoformans* and *Cryptococcus gattii*, are the causal agents. These are encapsulated basidiomycete found in the soil, tree bark and bird droppings. The spores or yeast form of fungus can easily be inhaled localizing in the alveoli space (37). M1 macrophages, TH1 and TH17 cells are essential in clearing infection (143). Although the immune response is sufficient to silence *Cryptococcus* to its dormancy, it is not sufficient to completely sterilize the lung (37, 143). Breakdown of immune defense leads to re-activation of this dormant stage and escape from lung, disseminating to other organs, particularly the brain (144). In immunocompetent individuals, *Cryptococcus* is thought to cause sub-clinical or asymptomatic pulmonary infections. This is often observed in urban areas that are densely populated and tend to have large pigeon colonies that live side-by-side with humans. Sub-clinical disease correlates with the presence of TH2 cells, M2 macrophages, and related cytokines and are detrimental in controlling dissemination and often associate with worse outcomes (38, 143, 145, 146). A few studies done in Northern America in Bronx area suggested that more than 50% of children over the age of 2 years were exposed to Cryptococcal antigens and their sera reacted to more than six antigens (147, 148). *Cryptococcus* exposure is associated with increased risk of developing asthma in children (147, 149). There is further evidence in mouse and rat animal models, where *Cryptococcus* infection exacerbates TH2 allergic airway inflammation (150). *Cryptococcus* virulent factors such as chitosan, proteases (Mpr1), laccases are the likely culprits in the development of TH2- type allergic asthma (**Table 1**), through favoring a defective immune arm (37). Encapsulation has been shown not to be essential in the initiation of TH2 type allergic asthma, despite its immune modulatory effects in IL-10 production and TH1 polarization (150, 151). Cryptococci cell wall is covered with chitin, which is known to have TH2-type stimulatory effects (37, 38). Human and mouse, both secrete Acidic Mammalian Chitinases (AMCase) known to digest huge chitin polymers and mediate TH2 airway immune responses (149, 152–154). Chitinases expression is increased in lung biopsies of adult asthmatics (152), although other studies have not shown comparable levels in BAL fluid (153). Children showing signs of exposure to Cryptococcal antigens, have elevated chitinases activity in the BAL fluid (149). A case study of a 10 year old boy also in the Bronx area who was sensitized to multiple fungal species including *C. neoformans* showed improvement in asthma symptoms such as reduced IgE and lung function when treated with itraconazole (126). Long term prospective studies are required to link early *Cryptococcus* exposure or infection to poor lung function and early acquisition

of allergic asthma in children. What is currently known is that children are exposed to Cryptococcal antigen after the 2 years of life and make antibody responses to at least six antigens of similar nature to the ones observed in rat sera infected with *C. neoformans* (147, 150). The nature of these antigens is unclear and whether they can be used as diagnostic markers particularly for skin-prick tests or CAP to test for *Cryptococcus* sensitization. These studies should be strongly supported by experimental model studies to determine which Cryptococcal antigens are essential in establishing allergic asthma at an early age.

Fungal skin sensitization is common particularly in atopic dermatitis (AD) patients. AD patients are often pre-disposed to secondary allergic responses such as asthma. Atopic march is a term used to describe this progression from skin sensitization to allergic asthma (42). Here, we mention two fungal species that are mainly found colonizing the skin surface and often associated with AD and have been associated with allergic asthma. We briefly, discuss the mechanisms on how these skin resident fungi can cause allergic asthma.

MALASSEZIA IN ATOPIC DERMATITIS AND ASTHMA

The skin serves as the first barrier to potential harm and is exposed to the largest number of pathogens due to its surface area, yet it contains the most diverse microbiome (155). Although large part of work has been focussed on bacterial species that colonize the skin such as *Staphylococcus epidermidis*, fungi accounts for almost a quarter of normal skin flora (156). *Malassezia* spp. colonize the skin of healthy and AD patients (156), but have also been found in sputum of asthmatic patients, which suggests their involvement in allergic asthma (35). *Malassezzia* spp. are dimorphic, lipophilic skin resident fungi that belongs to the phylum Basidiomycota, with at least 14 species identified so far in human and animal skin (36). The distribution of the species is geographically determined and largely driven by environment for example *Malassezia furfur* is more prevalent in Japan, whereas *Malassezia globota* and *Malassezia restricta* are found in Europe and US (36). *Malassezia* colonizes the skin as early as 0 and 1 days after birth and increases with time to almost 90% of skin being colonized by teenage years. However, there is considerable discrepancies in the species colonization between different studies (157, 158). A study by Lee et al. showed *M. restricta* to be common in teenagers and *M. globosa* predominant in adults over 50 years, whereas Gupta et al. found *M. globosa* to be more prevalent in younger subjects. *Malassezia* spp. is associated with at least three skin conditions including *Malassezia folliculitis*, Seborrheic dermatitis, *Pityriasis versicolor*, psoriasis and AD. The mechanisms on how *Malassezia* spp. contribute to any of these diseases are less defined and it is unclear if *Malassezia* spp. are the root causes of these conditions. In AD, *Malassezia* spp. is implicated as an aggravating factor, where it is thought that it releases allergens rather than being an infectious agent. The mechanisms of how *Malassezia* or its allergens promotes pathophysiology in AD patients is not well-defined, but is thought to be due to dysfunctional skin barrier, environmental factors, and other disturbances of normal skin flora, due to immunosuppression that favor mycelia growth over yeast form (36, 159). Once *Malassezia* spp. allergens enter the disrupted skin barrier, they are recognized by keratinocytes, Langerhan cells and other dermal dendritic cells that recognizes allergens and transport to local draining lymph nodes. This results in TH2 polarization and secretion of IL-4, IL-5, IL-13, and IgE which migrate back to the sight of inflammation further exacerbating AD. Other mechanisms of sensitization that have been suggested, are trafficking of *Malassezia* spp. allergens via extracellular vesicles (160). These extracellular vesicles (MalaEx) directly interact with keratinocytes and monocytes and are engulfed, becoming a major source of allergens, further exacerbating AD (**Table 1**) (160). *Malassezia* spp. sensitization (observed by *Malassezi*a specific IgE skin prick test) is less frequent in children compared to adults (161). *M. sympodialis* is the most frequently isolated species in AD skin lesions and patients on topical or systemic antifungal treatment show reduced severity of skin symptoms and IgE responses (161, 162). Both toll-like receptors (TLR-2 and TLR-4) and C-type lectin receptors, Mincle and Dectin-2 have been shown to bind specific ligands in *Malassezia* spp. and inducing specific pro-inflammatory cytokines in mouse macrophages (163, 164). However, these studies have not definitely shown whether these innate receptors promote *Malassezia*-specific IgE or promote IgE -independent skin inflammation that worsens AD.

TRICHOPHYTON INTERDIGITALE IN ASTHMA

Trichophyton interdigitale is a dermatophyte that can cause cutaneous infections when absorbed through the skin in millions of people worldwide. *Trichophyton* can also be inhaled, although it is unable to colonize the lung, it is associated with severe form of asthma (39). In some studies *T. interdigitale*-specific IgE responses correlate with severity of asthma and history of sensitization suggests childhood exposure (165, 166). *Trichophyton* and fungal skin infections are more common in immunocompromised people such as those with HIV infection, those receiving systemic corticosteroids and diabetes mellitus (39). In an African setting, there is significantly more people who are immunocompromised due to HIV and diabetes mellitus and develop skin infections, which may influence sensitization to *Trichophyton*. *Trichophyton* infections particularly scalp ringworm are predominant in children of African Caribbean or African American descent (167). In Africa, epidemiological data is scarce, however, these infections are likely to be high and probably involve other species of *Trichophyton* not reported in European, North American and other parts of the world. This early exposure to *Trichophyton* spp. certainly has profound impact on sensitization and development of allergies.

The immune responses to *Trichophyton spp.* is not well-defined and has somewhat remained an enigma with some anecdotes regarding its onset (39). It is believed that *Trichophyton* antigens are absorbed through barrier defected skin and these

antigens are then taken up by local Langerhan antigen presenting dendritic cells. These antigen presenting cells then migrate to local draining lymph nodes and present antigens to T cells (39, 165). There are two main dominant immune responses to *Trichophyton* spp., an immediate hypersensitivity (IH) and delayed hypersensitivity (DHT) (39, 168). *Trichophyton*-specific IgE and IgG4 and mast cell activation are the main characteristics of IH and also involve urticaria and flare and these are generally associated with TH2 cell activation and cytokines such as IL-4, IL-5 and are localized (166). DHT response usually takes 48 h to come up and is correlated with presence of TH1 cells and IFN-γ production by these activated cells (168). What is intriguing about immune responses to *Trichophyton* is that some allergens can drive both IH and DHT and stimulate both TH2 and TH1 cell subsets (168). What also remains elusive is how *Trichophyton* skin sensitized individuals develop allergic airway asthma. Atopic march is a common phenomenon in allergic diseases and is likely at play where the skin colonizers become an important source of chronic exposure. However, this has not been investigated in *Trichophyton* sensitized individuals with asthma symptoms and animal models have not been investigated, where an allergen, APC and T cell can be tracked *in vivo*.

CONCLUSIONS

Fungal species are ubiquitous, occupying at least 25% of Earth's biomass and are possibly found in every single niche of the human body cohabiting happily and forming part of the mucosal commensals. What is clear is that although the human body has learnt to tolerate fungi in most individuals, there are instances where this tolerance is dysregulated and cause various allergic pathologies. There is a lack of tools to detect fungal associated allergic sensitization and SPT for fungi are not routine. The use of artificial intelligence to predict IgE specific fungal allergens will likely have a bigger impact in hospital settings, particularly in prospective birth cohorts (169). In low- to middle-income countries, especially in Africa, these tools are currently beyond reach. Treatment options using both corticosteroids and antifungal drugs are promising but require further investigations. Antifungal drug access is also not widespread with some drugs either with no cheap generics, not available at all or only prescribed for life-threating infections such meningitis (170). Pharmacokinetics and drug-drug interactions between antifungal drugs and corticosteroids are not clear and require more randomized clinical trials in different population and age groups (120). Current FDA approved monoclonal therapy (omaluzimab or mepoluzimab) might be helpful in certain cases of severe hyper IgE, however, these are beyond reach for many African countries, so antifungal therapy remains the only attractive treatment method for fungal asthma (7, 120). It is important to note that only two countries in Africa have guidelines for asthma management in children, let alone fungal asthma (7, 171). Current SPT test in government hospitals do not include fungal allergens which makes it even harder to diagnose these asthma cases. There are limited studies focusing on the early development of fungal induced asthma and more work is needed to combine what is seen at clinics and experimental models. Long term birth cohort prospective studies are a valuable source of information regarding host genetics-environmental impact in the development of asthma (10, 172). In Africa, prospective birth cohorts to determine risk factors for asthma development are scarce (173, 174). More of these types of studies are needed to comprehensively evaluate impact of changing environment in the steady increase of asthma in Africa. Birth cohorts together with aerodynamic monitoring tools such as low-pressure impactor (ELPI) to specifically determine fungal allergen burden in indoor and outdoor environments will accelerate research in an African context and determine specific triggers of poor lung function and avoid a catastrophe (175).

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SH and FB wrote the manuscript.

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The Rise of *Coccidioides*: Forces Against the Dust Devil Unleashed

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Coccidioidomycosis (Valley fever) is a fungal disease caused by the inhalation of *Coccidioides posadasii* or *C. immitis*. This neglected disease occurs in the desert areas of the western United States, most notably in California and Arizona, where infections continue to rise. Clinically, coccidioidomycosis ranges from asymptomatic to severe pulmonary disease and can disseminate to the brain, skin, bones, and elsewhere. New estimates suggest as many as 350,000 new cases of coccidioidomycosis occur in the United States each year. Thus, there is an urgent need for the development of a vaccine and new therapeutic drugs against *Coccidioides* infection. In this review, we discuss the battle against *Coccidioides* including the development of potential vaccines, the quest for new therapeutic drugs, and our current understanding of the protective host immune response to *Coccidioides* infection.

Keywords: *Coccidioides*, valley fever, fungal vaccines, antifungal drugs, immunity

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INTRODUCTION

The *Coccidioides* genus contains *C. immitis* and *C. posadasii*, the etiological agents of Valley fever. This neglected disease occurs primarily in the southwestern United States, most notably in California and Arizona; however, cases have appeared in Washington pointing to an underappreciation of the geographic distribution of this organism (1, 2). Furthermore, cases outside the United States have been occurring in the northern region of Mexico (3) and areas of Central and South America (4, 5). *Coccidioides* is considered both a primary and opportunistic fungal pathogen occurring in both immunocompetent and immunocompromised individuals causing a spectrum of coccidioidomycosis. Most cases (~60%) are asymptomatic. For the remainder, pulmonary symptoms from underlying acute or progressive pneumonia are the most common reason patients seek medical help (6, 7). Additionally, dissemination can occur affecting a multitude of organs (**Figure 1**) and lead to the most severe complication, coccidioidal meningitis. Originally, the literature stated that an estimated 150,000 infections occur each year in the United States, and about 1% lead to disseminated disease with a third of those being fatal (7). Host factors strongly influence risk of disseminated disease such as immunosuppression, third trimester of pregnancy, old age, and ethnicity (i.e., African Americans and Filipinos) (6–12). Additionally, host response to treatment varies, current antifungals cause potential adverse side effects, and resistance to antifungals has recently become a concern (13, 14). Furthermore, infections caused by *Coccidioides* are on the rise (15), and new estimates of the annual number of new U.S. infections are more

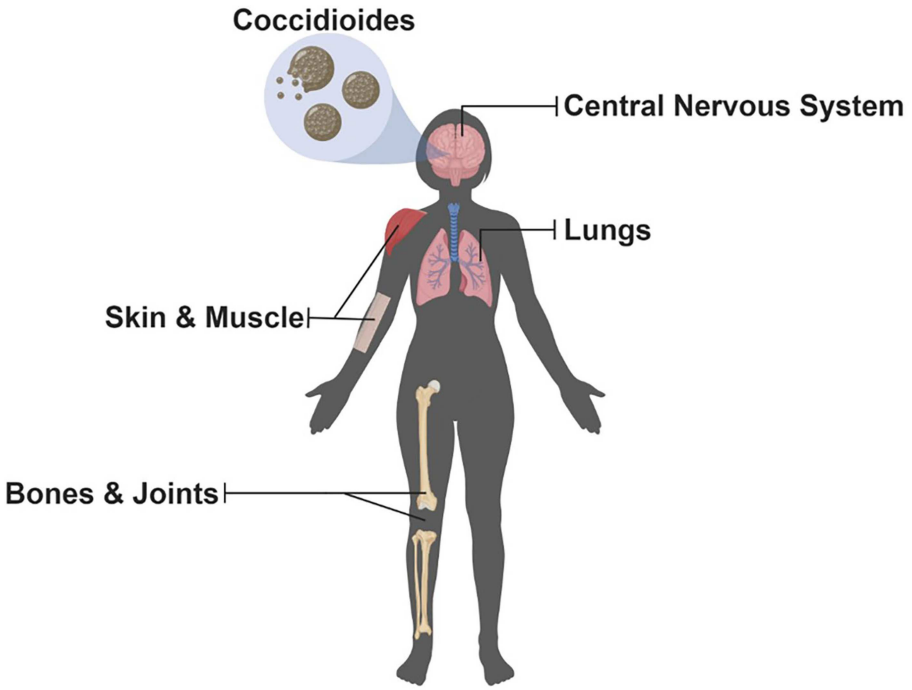

FIGURE 1 | Potential Organs Infected by *Coccidioides*. Since inhalation is the most common route of infection, the lungs are the most common organ that becomes infected with *Coccidioides*; however, dissemination can occur allowing for multiple organs, highlighted above, to become infected but are uncommon (Illustration created with BioRender).

than twice (350,000) that of previous estimates (16). The reason for the increase in *Coccidioides* cases is largely unknown; however, factors such as changes in the environment and surveillance methodology could be contributing factors (15). Taken together, there is an urgent need for new antifungal agents, a better of understanding of host response to infection, and the development of a vaccine to combat coccidioidomycosis. Here, we review the current understanding of the host immune response to infection and protection, advances in drug development, and discuss promising approaches to developing a *Coccidioides* vaccine; a one stop-shop to understand current research in the battle against the Dust Devil.

HOST IMMUNE RESPONSE TO *COCCIDIOIDES*

Coccidioides grow in the soil as fungal mycelia which segment into arthroconidia (spores) that can then become aerosolized, inhaled, and cause infection. Once a host is infected, arthroconidia transition into mature rupturing spherules within 5 days of infection (17). Therefore, during the early days of infection, morphological variation of *Coccidioides* is high as the organism is switching from arthroconidia to its parasitic stage, the spherule. In this section, we will discuss what is known about the host immune response to *Coccidioides* infection, first focusing on the early immune response, and then discussing the protective host immune response to battle coccidioidomycosis.

Early Innate Immune Response to *Coccidioides*

The innate immune response is the first line of defense against fungal pathogens and clearance relies heavily on phagocytosis by macrophages and neutrophils (18). Phagocytosis can occur on inhaled arthroconidia (3–5 μm) (17) and endospores (2–7 μm); however, mature spherules are too large (15–80 μm) (19) and phagocytic cells fail to engulf these fungal organisms (20). Neutrophils for example can only partially engulf cells that are about 11 μm (21) which is below the threshold of the size of a mature spherule. Studies have shown an influx of neutrophils during infection with *Coccidioides* (22) and when spherules burst releasing hundreds of endospores (23, 24). Past reports have shown C57BL/6 mice depleted of neutrophils are as susceptible as wild-type mice when infected with wild type *Coccidioides* (19). Conversely, when mice are vaccinated with a live-attenuated strain of *Coccidioides* (ΔT, genetically engineered mutant originally designated *Δcts2/ard1/cts3*), protection relies on the presence of neutrophils (19). Additionally, studies conducted by Gonzalez et al. showed that mice deficient in NADPH oxidase (NOX2) were more susceptible to infection with *C. posadasii* compared to wild type mice while inducible nitric oxide synthase (iNOS) knock-out mice demonstrated that iNOS does not play a significant role in the control of *Coccidioides* infection (25, 26). Interestingly, $NOX2^{-/-}$ mice had substantially more infiltration of neutrophils in the lungs compared to wild type mice while $iNOS^{-/-}$ mice had a significant increase of neutrophils at day 7 but not day 11 post challenge. Overall these

studies demonstrate that neutrophils play a role in the proper inflammatory response during a *Coccidioides* infection, and dysregulation of an inflammatory response can be detrimental to the host.

Coccidioides spherules can also escape phagocytosis from macrophages (23). Macrophages vary in size depending on location in the host: 5 μm spleen, 10 μm peritoneal surface, and 15 μm alveoli (27). Studies have demonstrated an evolutionary conserved particle/pathogen size ratio contributes to pathogen clearance and recognition (28), suggesting the inability of macrophages to phagocytose mature spherules. Vaccination studies have demonstrated the influx of macrophages to the lungs of vaccinated mice compared to unvaccinated mice after challenge with *Coccidioides* (22). However, the role of macrophage subsets (i.e., classically and alternatively activated macrophages or M1 and M2 macrophages) in the protective host immune response against *Coccidioides* has yet to be elucidated. Studies have shown that mouse peritoneal macrophages stimulated with *Coccidioides* spherules produce tumor necrosis factor alpha (TNF-α) (29). Furthermore, studies have shown increases in cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin (IL)-17 in mononuclear cells from bronchoalveolar lavage fluid (BALF) from patients with pulmonary coccidioidomycosis (30).

Studies further determined which pattern recognition receptors (PRRs) on peritoneal macrophages were important for recognition of *C. posadasii* spherules. Using peritoneal macrophages from wild-type cells compared to different knockout mice (i.e., $TLR2^{-}/^{-}$ and $MyD88^{-}/^{-}$), results demonstrate the response to spherules is dependent on Toll-like receptor 2 (TRL2), myeloid differentiation factor 88 (MyD88), and Dectin-1 (31). Dectin-1 is a C-type lectin receptor shown to interact with components of the fungal cell wall. Studies have shown the importance of this C-type lectin receptor where $Dectin1^{-}/^{-}$ mice infected with *Coccidioides* demonstrated increased pulmonary fungal burden and decreased Th17 cytokines (32). Studies further suggest that increased susceptibility of C57BL/6 mice to coccidioidomycosis is due to alternative splicing of the Dectin-1 gene (33). Furthermore, studies have identified that null mutations in Dectin-1 predispose hosts to chronic mucosal candidiasis (34). Additionally, people with mutations in the *CARD9* gene, Dectin-1, and other C-type lectin receptors signaling through this gene have increased susceptibility to fungal infections (35). Another C-type lectin receptor, the mannose receptor, has been shown to be important in the immune response of human coccidioidomycosis but does not play a role in a murine model of coccidioidomycosis (36–38). Studies demonstrated an association with low mannose-binding lectin (MBL), a collectin that is part of the innate immune system, serum levels among patients exhibiting an active *Coccidioides* infection compared to otherwise healthy individuals; however, the role of MBL in the pathogenesis of *Coccidioides* has yet to be determined (37). Recent studies further investigated the role of multiple receptors that use MyD88 to determine which of these receptors are required for resistance against coccidioidomycosis. Of all the surface receptors investigated, results from the studies determined IL-1R1 signaling to be important for protection against coccidioidomycosis (39). Overall, these studies demonstrate the potentially crucial role of C-type lectin receptors and certain TLRs to protect against coccidioidomycosis, but much remains to be done.

Dendritic cells (DCs) act as a bridge between the innate and adaptive immune response. DCs initiate the immune response by capturing antigens and then activate and modulate lymphocytes. Mature DCs have the ability prime naïve T cells toward phenotypes (Th1 and Th17) protective against coccidioidomycosis (discussed below) (40). Studies have demonstrated that DCs pulsed with *Coccidioides* antigen (spherulin, spherule lysate) can activate DC maturation and lymphocyte proliferation in non-immune individual cells (41). Furthermore, studies investigated the effects of DCs pulsed with a coccidioidal antigen preparation, T27K, using PBMCs from patients with disseminated coccidioidomycosis compared to healthy individuals (42). Results from these studies demonstrate that DCs can be generated by patients with disseminated coccidioidomycosis, and stimulation with T27K led to increased IFN-γ levels in both disseminated and healthy patient samples. Furthermore, studies have demonstrated that suppressing DC responses led to defective T cell responses. BALB/c mice are highly susceptible to infection with *Coccidioides*, whereas DBA/2 mice are more resistant. Bone-marrow derived DCs (BMDCs) from DBA/2 mice infected with *Coccidioides* demonstrated an increase in IL-12 secretion and T cell co-stimulatory cell surface molecules compared to BALB/c mice (43). Thus, these studies suggest BALB/c mice could be more susceptible due to impaired DC responses; however, more studies are needed using other mouse strains that are susceptible to infection with *Coccidioides*.

Despite species divergence of *C. immitis* and *C. posadasii* about 5.1 million years ago (44), many studies state that these two species cause similar disease clinically. However, studies from our laboratory allude to differential early host innate responses among species of *Coccidioides* in a murine model of coccidioidomycosis (45). Since host responses strongly influence clinical disease, differences in the first line of defense against coccidioidomycosis could attribute to differences in outcome of disease. Mice were infected with 1×10^5 arthroconidia of either a *C. immitis* pure strain (2006), *C. immitis* hybrid strain (RS), or a *C. posadasii* pure strain (Silveira). Real-time RT-PCR analysis of mouse lungs shows differential responses across strains. Expression of proinflammatory cytokine levels (IL-1α and IL-17α) were significantly increased in the mice infected with the 2006 strain (*C. immitis*) at day 5 post infection compared to all other infected mice. Silveira (*C. posadasii*) infected mice demonstrated an increase in proinflammatory cytokine IL-1β at day 1 post infection and immunoregulatory cytokine IL-10 at day 5 post infection compared to other strains (45).

Coccidioides has other means of avoiding phagocytosis and evading the immune response. Spherule outer wall glycoprotein (SOWgp) is a major antigen present on the cell surface of *Coccidioides* (46, 47). This glycoprotein is highly expressed during the transition to spherules, and demonstrates immunogenic properties (46, 47). Interestingly, studies have shown that a specific metalloproteinase (Mep1) is secreted during endosporulation, which then digests SOWgp to prevent

host recognition (48). Furthermore, mice vaccinated with recombinant SOWgp and then challenged with a *C. posadasii* strain with the *MEP1* gene disrupted demonstrated increased survival compared to the parental or revertant strain (48). Other studies have demonstrated that *Coccidioides* can suppress nitric oxide (NO) production in macrophages; however, these studies show NO is not critical for *in vitro* killing of *Coccidioides* (49). Although these studies give us insights into *Coccidioides* pathogenesis, more studies are needed to understand the immune evasion strategies of this pathogen.

Protective T-Cell Host Immune Response to Combat *Coccidioides*

Results from both clinical data and mouse models of coccidioidomycosis have demonstrated that T cell immunity is crucial for protection against coccidioidomycosis. Additionally, deficiency in $CD4^+$ T cells results in increased susceptibility to infection with *Coccidioides* (50). $CD4^+$ T cells can differentiate into distinct lineages that produce certain cytokines in response to a pathogen. Cytokines such as IL-12 and IFN-γ are associated with T cell helper 1 (Th1) responses, which has been shown to be important for protection in mouse models of coccidioidomycosis (51, 52) and *in vitro* studies using human PBMCs (53). Additionally, patients with IL-12 and IL-1 receptor deficiencies demonstrate increased dissemination of *Coccidioides* (54, 55). A Th2 immune response is activated by cytokines such as IL-4 and IL-5 and has been shown to downregulate the host immune response during infection with *Coccidioides* (51). On the other hand, these cytokines can induce B cell responses which have been shown to play a role in protection in a mouse model of coccidioidomycosis (51, 56, 57). However, the role of Th2 and antibodies in the clearance of *Coccidioides* has yet to be resolved and requires further study. Additionally, the detection of anti-*Coccidioides* antibodies for the diagnosis of coccidioidomycosis is not reliable in humans (58). Recently, the role of Th17 responses which produce proinflammatory cytokines such as IL-17 and IL-22 has been investigated (59). Vaccination studies by Hung et al. demonstrate the critical role of Th17 responses in protection against coccidioidomycosis (22). In these studies, mice lacking the IL-17 receptor that were vaccinated with the ΔT strain were highly susceptible to challenge with *Coccidioides*. Furthermore, mice deficient in IFN-γ and IL-4 receptors were still protected against challenge with *Coccidioides* equivalent to wild-type mice. Thus, demonstrating conflicting results of the importance of IFN-γ in the protection against coccidioidomycosis. These studies also demonstrate the immune response of ΔT vaccinated mice challenged with *Coccidioides* is a mixed Th1, Th2, and Th17 response (22). Overall, studies demonstrate that each of these subsets play a role in the protection against coccidioidomycosis.

Along with $CD4^+$ T cells, mouse studies show that $CD8^+$ T cells play a role in protection against infection with *Coccidioides* (60). Studies have shown an increased percentage of $CD8^+$ T cells were present post challenge among ΔT vaccinated mice compared to non-vaccinated mice (22). Importantly, BALF from patients with coccidioidomycosis demonstrated an increased proportion of $CD8^+$ T cells in patients with acute pulmonary *Coccidioides* infection compared to all other groups (30). Additionally, studies have shown that $CD8^+$ T cells can compensate for the lack of $CD4^+$ T cells and confer protection against fungal pathogens (60–63). Studies analyzing pediatric patients with coccidioidomycosis demonstrated an overall lower adaptive immune response in persistent disease patients with a trend toward lower $CD4^+$ and $CD8^+$ T cells, and significantly fewer B cells compared to control and resolved patients (64). Additionally, these studies found no difference in Th1 frequencies among patient populations; however, patients with persistent disease had a lower frequency of Th17 and higher T regulatory (Treg) frequencies compared to patients with resolved disease. Therefore, studies from both human and mouse models of coccidioidomycosis have demonstrated an association between increased Th17 responses and resolution of infection.

DEVELOPMENT OF A *COCCIDIOIDES* VACCINE

Despite earnest efforts, there is currently no clinically available vaccine against any fungal organism; although, early results have been favorable in the development of a *Candida* vaccine (65). The overall goal of an anti-coccidioidal vaccine is to prevent disease. Immunization against coccidioidomycosis appears possible since patients who have recovered from an initial coccidioidal infection rarely become ill from a second infection and additional exposure (66). The first experimental anti-*Coccidioides* vaccine developed was the formalin-killed spherule (FKS) vaccine that demonstrated promising results in mice (67). However, human trails established no differences between FKS-vaccinated group and the placebo group (68). Additionally, the FKS-vaccinated group experienced severe side effects at the local injection site. Herein, we discuss various strategies to develop a vaccine to combat coccidioidomycosis.

Live Attenuated Vaccines

Live attenuated strains have proven to be successful in stimulating the immune response similar to a naturally occurring infection (69–72). However, an ideal vaccine candidate needs to have an impeccable safety profile in all populations such as the immunocompromised (73). Although a live vaccine may not be useful in a human clinical setting, understanding the protective host immune response against *Coccidioides* is imperative to design a suitable and effective recombinant vaccine to combat coccidioidomycosis. For example, chitinase activity in *C. posadasii* was inhibited by disrupting two chitinase genes (*CTS2* and *CTS3*) and a third gene contiguous to *CTS3*, to obtain an attenuated mutant that was no longer able to endosporulate, Δ*cts2/ard1/cts3* (51). This genetically engineered strain demonstrated protection in mice against coccidioidomycosis and is now designated as the ΔT vaccine strain (51, 74). Using this vaccine strain, studies have demonstrated the important parameters for eliciting a protective host immune response against coccidioidomycosis. As discussed above, the ΔT vaccine helped to elucidate the important role of $CD4^+$ T cells,

particularly Th1 and Th17 immune responses that are critical for protection (22).

More recently, a homolog of the gene *CPS1*, a virulence factor found in a maize pathogen (75), was deleted in a strain of *C. posadasii* (76). This deletion resulted in essentially complete attenuation of pathogenicity in both wild type and immunodeficient mice. Furthermore, mice vaccinated with live ΔCPS1 were protected against an otherwise lethal infection with wild type *C. posadasii* and *C. immitis* (76, 77). Further studies demonstrated a primarily Th1-type response in mice vaccinated with ΔCPS1 and challenged with wild-type *C. posadasii* compared to unvaccinated mice (77). Both the ΔT and ΔCPS1 strains are vital tools needed to determine the protective host immune response needed to battle *Coccidioides*. Interestingly, both of these mutant strains undergo initial spherulation in the host before arresting growth.

A practical attraction of a live attenuated *Coccidioides* vaccine candidate is that manufacturing costs to make a clinically feasible product should be low. Production costs have been a road block for an earlier recombinant vaccine (78); however, as with any live vaccine, safety is a critical consideration. Since ΔCPS1 is a complete gene-deletion, reversion is hard to imagine. On the other hand, new mutations in other genes might compensate for the missing gene and result in gain-of-function and cause disease, especially in more immunosuppressed individuals (73, 79). ΔCPS1 is currently being developed as a live vaccine candidate to prevent Valley fever in dogs (80). Should this prove successful, it would provide a proof-of-concept supporting further development to prevent Valley fever in humans. The exact path for this vaccine candidate to humans has yet to be determined. There is no precedent since a live attenuated eukaryotic vaccine has yet to be given FDA approval. Furthermore, the market for a vaccine to prevent Valley fever is relatively small. While there is a very strong public case for preventing this disease (81), it is much more challenging to make a business model with a return on investment competitive with other opportunities for investors. It is likely that a Valley fever vaccine will only be developed if public resources, state or federal, are deemed appropriate for this purpose.

Novel Adjuvants and Protein Vaccines

A safer alternative to attenuated vaccines is the use of recombinant proteins; yet, these may require an adjuvant to strengthen the immune response and optimize efficacy (82). Studies sought to characterize a novel adjuvant, a peptide agonist of the biologically active C-terminal region of human complement C5a referred to as EP67, conjugated to the live ΔT vaccine strain (83). These studies found that BALB/c mice immunized with the EP67-conjugated vaccine demonstrated increased survival rates and reduced fungal burden compared to the non-conjugated vaccine. Additionally, mice given the conjugated vaccine had increased infiltration of macrophages and DCs by day 7 post challenge while neutrophil numbers were decreased by 11 days post challenge compared to the non-conjugated vaccinated mice. Furthermore, the novel adjuvant EP67 increased Th1 and Th17 immune responses; therefore, augmenting T cell immunity and enhancing protective efficacy of the live ΔT vaccine strain (83).

Early studies suggest multivalent vaccines are more effective against coccidioidomycosis compared to a single peptide vaccine (84–86). Early studies introduced rAg2/Pra as a potential vaccine candidate; however, varying routes of challenge led to conflicting results (87, 88). Thus, improved protection efficacy against *Coccidioides* infection in mice by adding an *Coccidioides*-specific antigen (CSA) to the rAg2/Pra were completed (84). The inclusion of another antigen Prp2, and development of a combined vaccination of rAg2/Pra+rPrp2, produced significantly improved protection compared to either of the recombinant proteins alone (89). Additionally, recent studies have demonstrated a Ag2/Pra-specific response in mice using a DC-based vaccine which was prepared by transfecting primary bone marrow-derived DCs with a plasmid encoding Ag2/Pra (90). Prior studies demonstrated that the DC-based vaccine reduced fungal burden and increased IFNγ levels in the lung homogeneates from vaccinated mice compared to control mice (91).

Using two-dimensional gel electrophoresis and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), studies identified another protein, *PMP1* (peroxisomal matrix protein 1), which also demonstrated protection in a mouse model of coccidioidomycosis (92). Additional protective antigens that were used as potential vaccine candidates include *PEP1*, *PLB*, and *AMN1*, which demonstrated enhanced protection as a multivalent vaccine compared to a single antigen alone (85). An alternative approach to a multivalent vaccine to lower cost is the used of epitope-based vaccines (EBV) which has been shown to effectively induce an immune response, is relatively easy to produce, and expected to be safe to use in humans (93, 94). Studies conducted by Hurtgen et al. created a recombinant EBV (rEBV) which incorporated *PEP1*, *AMN1*, and *PLB* into a single epitope-based vaccine which was either admixed with an adjuvant or loaded into glucan particles (GPs) (95). Overall, these studies demonstrated that the rEBV plus GP vaccination was superior to all formulations tested in this study showing enhanced survival, reduced fungal burden, and robust Th1 and Th17 immune responses compared to control mice with GPs alone. GPs are purified, hollow, porous yeast cell-wall particles derived from *Saccharomyces cerevisiae*. There have been several types of yeast particles created for vaccine development (96).

Recently, studies created a recombinant chimeric polypeptide antigen, rCPa1, that consist of Ag2/Pra, Cs-Ag, Pmp1, and 5 T cell epitopes from *PEP1*, *PLB*, and *AMN1* from *C. posadasii* (97). Additionally, they tested the efficacy of rCpa1 encapsulated in differently formulated yeast cell-wall particles. These studies identified a promising vaccine candidate, rCpa1, encapsulated in glucan-chitin particles (GCP-rCpa1) that showed increased survival, significantly reduced fungal burden, and a mixed protective Th1 and Th17 response (97). Additionally, recent studies conducted by Hayden et al. demonstrated that mice immunized with recombinant Ag2 expressed in maize and loaded into GCPs had reduced fungal burden in *Coccidioides* challenged mice similar to Ag2 derived from *Escherichia coli* (98).

Furthermore, oral administration of Ag2 fused onto a DC carrier peptide (DCpep) demonstrated protective Th17 responses. More studies are needed to characterize these new vaccine candidates to determine if clinical trials are on the horizon. To move into clinical trials, we need to test potential vaccine candidates in multiple animal models including transgenic mice expressing human receptors.

CURRENT TREATMENTS AND DRUG DISCOVERY

Coccidioidomycosis represents a spectrum of illness ranging from asymptomatic acquisition with resultant immunity to severe and life-threatening disseminated infections. Even in otherwise uncomplicated primary pulmonary infection the symptoms of fever, chills, cough, joint pain, and malaise can last weeks to months (99). Severe cases including dissemination to the skin, bone, or brain (**Figure 1**) can be difficult to treat and in some cases require life-long antifungal therapy. Currently, the most common management of coccidioidomycosis includes antifungal agents such as fluconazole or itraconazole; however, guidelines suggest an individualized approach to patient management (13). Nevertheless, new concerns of toxicities and side effects, with either acute or long-term use, caused by these agents have seen renewed interest in the development of new agents to combat this disease. Herein, we discuss briefly the current and future treatment options for patients with coccidioidomycosis. A comprehensive review of current treatment options against coccidioidomycosis has recently been published (100).

Polyenes

Amphotericin B has been a widely used agent in the treatment of coccidioidomycosis over the last 50 years (101) and is currently available in multiple intravenous formulations: amphotericin B deoxycholate (AmBd), liposomal amphotericin B (L-AMB), amphotericin B colloidal dispersion (ABCD), and amphotericin B lipid complex (ABLC) (**Table 1**) (100). Overall, these formulations are met with adverse effects such as nephrotoxicity, hypokalaemia, phlebitis, fever, chills, hepatotoxicity, and anemia (102–105). Historically, long courses of amphotericin B therapy was prescribed in an attempt to provide curative therapy given the lack of an orally available efficacious agent. With the availability of the less toxic triazole antifungals, amphotericin B therapy is reserved for the treatment of patients who are intolerant or refractory to the other available antifungal agents or those with severe disease.

There have been numerous studies using animal models that have demonstrated the efficacy of the lipid formulations of amphotericin B therapy against coccidioidomycosis (106–109). Although clinical studies have been sparse, the use of amphotericin B against multiple forms of coccidioidomycosis has demonstrated its efficacy. A retrospective study demonstrated similar efficacy of ABLC and L-AMB in the treatment of severe coccidioidomycosis; however, L-AMB may be the preferred agent with less renal toxicity during treatment compared to ABLC (110). Studies have shown that coccidioidal meningitis treated with amphotericin B deoxycholate via the intrathecal route demonstrates a much more successful treatment compared to the intravenous route (111). However, discussion with those experienced in the treatment of intrathecal therapy is highly recommended if intrathecal therapy is needed during clinical care given the potential morbidity with treatment via this approach (112, 113).

Triazoles

The triazoles used to combat coccidioidomycosis include: fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole. These triazoles prevent the conversion of lanosterol to ergosterol thus affecting ergosterol synthesis. More specifically, these agents, with significant affinity differences, inhibit cytochrome P450 (CYP)-dependent 14-α-demethylase (114). This affinity difference leads to variability among the antifungal agents in their efficacy, spectrum of activity, and side effect profile. Despite the commercial availability of the triazoles, few have been evaluated in prospective clinical trials due to the regional nature of the disease and the high financial burden of these types of studies. However, the designation of coccidioidomycosis as an orphan disease may facilitate these efforts and allow future antifungal agents to be fully evaluated in prospective fashion.

Fluconazole

Fluconazole is the most frequently prescribed antifungal agent and clinical guidelines suggest it to be a first line agent against multiple forms of coccidioidomycosis (13). Advantages of this agent include low cost, tolerability, the availability of both an oral and intravenous formulations, long half-life, and excellent bioavailability (see **Table 1** for an overview of benefits, weaknesses, and adverse effects) [for pharmacokinetics of antifungal agents see recent review article (100)]. Fluconazole has the ability to penetrate most tissues with adequate concentrations within the cerebrospinal (CSF) fluid allowing for the treatment of coccidioidal meningitis (CM) (13, 115). Although adverse effects from the use of fluconazole are largely benign, patients have experienced hepatotoxicity, heart corrected QT interval prolongation, alopecia, xerosis, and cheilitis (100, 116).

A recent study performed a large-scale susceptibility test to understand triazole minimum inhibitory concentrations (MICs) of *Coccidioides* isolates. These results revealed increased fluconazole MICs across multiple *Coccidioides* isolates tested ($\geq$16 μg/ml, 37.3% of isolates; $\geq$32 μg/ml, 7.9% of isolates) (14). This decreased *in vitro* susceptibility of fluconazole may explain the need for higher fluconazole doses during treatment of coccidioidomycosis (13) and a dose-dependent response to fluconazole has been observed using a murine model of systemic coccidioidomycosis (117); however, this *in vitro* data has yet to be correlated with clinical outcomes. At this time, no comparative trial has evaluated the dose-dependent response of fluconazole in a randomized study; although, efficacy has been definitively demonstrated (118). Recently, tolerability of long-term fluconazole therapy was assessed, and it was demonstrated that out of 124 patients ~50% had adverse effects (116). The most common adverse effects patients experienced included xerosis,

TABLE 1 | A brief overview of antifungal agents benefits, weaknesses, and adverse effects in the treatment of coccidioidomycosis.

Antifungal agent	Benefits	Weaknesses	Adverse effects
Triazoles			
Fluconazole	Low cost/tolerable	High MIC values *in vitro*	Hepatotoxicity, QTc prolongation, alopecia, xerosis, and cheilitis
Itraconazole	Highly efficacious and tolerable	CSF and bone penetration, TDM	Hepatotoxicity, gastrointestinal distress, hypertension, hypokalemia, negative inotrope, and peripheral edema
Voriconazole	High CSF penetration	Variable bioavailability and TDM	Hepatotoxicity, photopsia, and photoxic skin reactions, visual hallucinations, rashes/long-term use lead to skin carcinoma, alopecia, and xerosis
Posaconazole	Penetrates most body sites and effective against nonmeningeal coccidioidomycosis	Therapeutic drug monitoring advised, low/variable CSF penetration	Gastrointestinal distress, hypokalemia, hypertension, peripheral edema
Isavuconazole	Efficacious against primary coccidioidomycosis, prolonged half-life, and tolerable	Limited clinical data against meningeal coccidioidomycosis	Gastrointestinal distress and hypokalemia
Polyenes-Amphotericin B			
AmBd	Intrathecal route	Highly toxic	Nephrotoxicity, hepatotoxicity, hypokalemia, phlebitis, fever, chills, dyspnea, chest/back pain
ABCD	N/A		
ABLC	N/A		
L-AMB	Less renal toxicity		

AmBd, amphotericin B deoxycholate; ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; L-AMB, liposomal amphotericin B.

alopecia, and fatigue, which resulted in 65% of patients requiring a therapeutic change.

Itraconazole

Itraconazole is also frequently prescribed to treat coccidioidomycosis (13). This antifungal agent is available primarily as a capsule or oral solution (100). Advantages of using itraconazole include long half-life, efficacy, and tolerability (**Table 1**), although gastrointestinal side-effects are common with the oral solution, negative inotropic effects on cardiac output have been reported (119). Also, recent reports describe the development of hypertension following itraconazole initiation (120). However, the bioavailability is highly variable and studies have shown itraconazole to exhibit poor CSF (121, 122) and bone penetration (123). Additionally, due to variable bioavailability, therapeutic drug monitoring is recommended to ensure adequate absorption (124).

Despite poor CSF and bone penetration, studies have shown itraconazole to be highly efficacious in the treatment of both osseous coccidioidomycosis and coccidioidal meningitis (118, 125–127). Galgiani et al. compared fluconazole and itraconazole therapy in non-meningeal coccidioidal infections. These studies demonstrated an enhanced response in itraconazole treated patients compared to fluconazole treated patients with osseous coccidioidomycosis (118). Overall, they found itraconazole tended to be slightly more efficacious with fewer relapses compared to fluconazole treated patients. Studies using a murine model of CM demonstrated prolonged survival of mice infected with *Coccidioides* treated with either 50 mg/kg of itraconazole or fluconazole (125). At this same dose, they found equal clearing of fungi from both brain and kidney; however, itraconazole demonstrated an enhanced clearing of fungi in spinal cord and lungs.

Voriconazole

Voriconazole is often used for patients who are intolerant or refractory to other triazoles in the treatments of coccidioidomycosis (128, 129). The advantages of this antifungal agent include the availability of in both intravenous and oral formulations, high oral bioavailability, wide distribution throughout body, and the ability to penetrate the CSF (**Table 1**) (100). Nevertheless, voriconazole exhibits many attributes necessitating a working knowledge of its differences compared to other agents. Voriconazole possesses a variable half-life (patient dependent), many drug-drug interactions, hepatotoxicity, visual disturbances, rashes, alopecia, xerosis, and long-term toxicity concerns including cutaneous malignancy (129–134). Due to the variable half-life and the contraindication in patients with renal dysfunction, therapeutic drug monitoring is highly recommended (135).

The efficacy of voriconazole in the treatment of coccidioidomycosis has been demonstrated in retrospective series with favorable outcomes observed in the majority of reported cases including those with bone meningeal and non-meningeal disease (129, 130).

Posaconazole

Posaconazole was initially available only as an oral solution; however, bioavailability was a problem (136). Currently, an intravenous formulation and delayed release oral tablet are now

available and the latter demonstrates significant improvement of absorption (137). Posaconazole has been shown to penetrate most sites of the body, but exhibits poor CSF penetration (138, 139). Common adverse effects caused by posaconazole treatment include gastrointestinal distress, hypokalemia, hypertension, peripheral edema, dry mouth, and headache (140, 141). Additionally, there are concerns of potential toxicity with high posaconazole concentrations (142); therefore, therapeutic drug monitoring is suggested (**Table 1**) (143).

Studies have shown the efficacy of posaconazole for the treatment of coccidioidomycosis in murine models (144, 145). One study demonstrated that mice treated with 10 mg of posaconazole showed >70% sterilization in the spleens and livers of *Coccidioides* infected mice while itraconazole treated mice resulted in no sterilization in the same tissues tested (144). Clinically, posaconazole treatment has shown efficiency in the treatment of refractory cases of coccidioidomycosis (129, 140, 146, 147).

Isavuconazole

Isavuconazole exist as a prodrug, isavuconazonium sulfate, which is cleaved by plasma esterases into the active moiety. This novel triazole is available in both oral and IV formulations, has a prolonged half-life (∼130 h), high bioavailability, and is widely distributed through-out the body (**Table 1**). Additionally, isavuconazole has shown efficacy clinically against multiple disparate fungal pathogens including the endemic mycoses (148–152). Isavuconazole has been shown to cause adverse effects; the most commonly observed include gastrointestinal disorders (diarrhea and nausea/vomiting) and hypokalaemia (149).

Thus far, there is limited clinical data for the use of isavuconazole therapy on patients with coccidioidomycosis. A prospective study has demonstrated efficacy in the treatment of primary infection with *Coccidioides* (151) and a retrospective study has demonstrated the potential use of isavuconazole in coccidioidal meningitis in the salvage setting (153).

Combination Therapy

It stands to reason that targeting multiple pathways using a combination of drugs would improve efficacy. However, clinical trials are lacking in the case of combination therapy against coccidioidomycosis. Interestingly, studies using a murine model of coccidioidomycosis have demonstrated the synergistic effects of combination therapy with caspofungin and amphotericin B deoxycholate increasing survival and decreasing fungal burden of mice compared to monotherapy with either treatment (154). This is noteworthy as the echinocandins have little activity against *Coccidioides* species and should not be used as monotherapy or

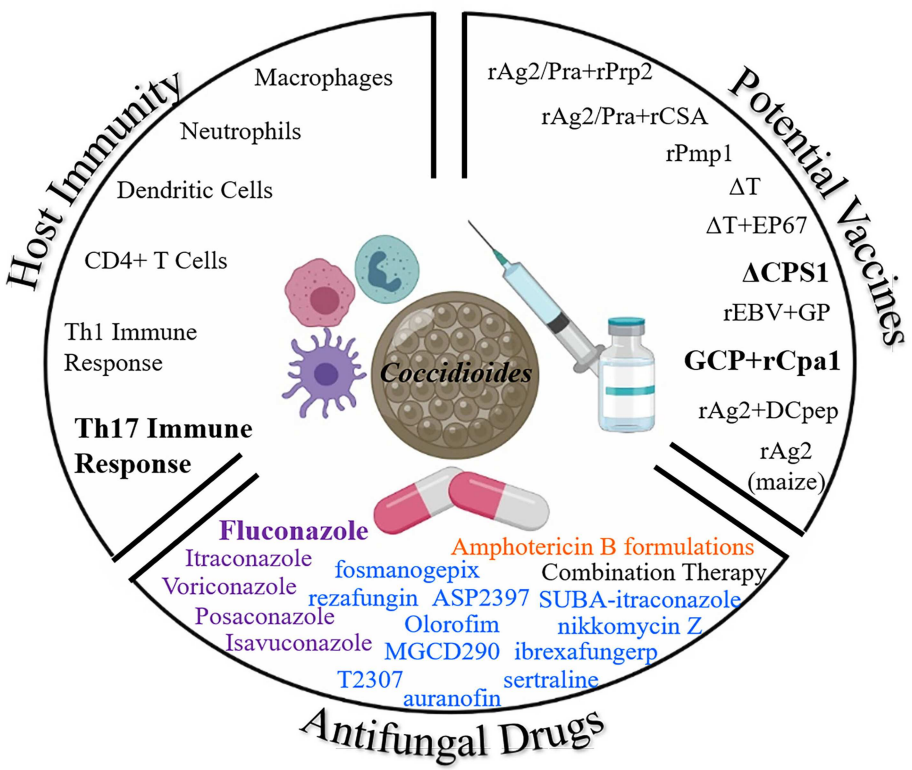

FIGURE 2 | Three Arsenals to Combat *Coccidioides*. Here we highlight the current battle against *Coccidioides* from antifungals, potential vaccines, and the protective host immune response. Bolded terms: important for host protection, most common antifungal drug, and most promising current vaccine candidates against coccidioidomycosis. Color coding for antifungal drug classes: purple, Azoles; blue, drugs in development; and orange, Polyenes (Illustration created with BioRender).

outside of the salvage setting. Additional reports on the potential utility of combination therapy against coccidioidomycosis is scant, and includes murine models of infection and a single case reports/case series in the salvage setting (111, 155–157). Overall, these cases demonstrate the potential promise of the use of combination therapy against refractory coccidioidomycosis.

New Drug Development

Although recent development of new and less toxic triazoles have been a welcome advance, there is a clear need for more effective and less toxic antifungal agents/therapies, particularly fungicidal oral agents. There are numerous agents currently in development with new modes of action and potentially reduced toxicity. A new formulation of itraconazole (SUBA-itraconazole) (158) has recently become available and clinical studies are ongoing. Novel amphotericin B formulations are currently in development (159). Additionally, some of the drugs in development exhibit broad-spectrum activity against multiple mycoses. Olorofim (formerly F901318) is an orotomide (inhibitor of dihydroorotate dehydrogenase) and has shown excellent *in vitro* activity against a number of fungal pathogens including *Coccidioides*, and murine models have suggested fungicidal activity (160) with a phase II clinical trial currently ongoing. Fosmanogepix (formerly APX001), a GPI-anchor inhibitor, has shown activity against a broad spectrum of fungal pathogens (161–166). A recent study evaluated the activity of prodrug APX001 and prodrug analogs against *C. immitis* and treatment with APX001 in *Coccidioides* infected mice resulted in significantly longer survival rates and reduced fungal burden than fluconazole or control treated mice (167). Another potential new drug, nikkomycin Z, a chitin synthase inhibitor, is nearing phase 2 clinical trials (168) and has shown similar promise in murine models of infection (169). Also in development are new glucan synthase inhibitors [rezafungin and ibrexafungerp (formerly SCY-078)] (170), a fungal mitochondrial inhibitor (T2307), and a histone deacetylase inhibitor (MGCD290), some with an unknown mode of action (ASP2397), and some repurposed from cancer therapy (sertraline and auranofin) (100, 159, 171, 172).

CONCLUSION

Due to the rise of *Coccidioides* infections and concerns regarding toxicity of current antifungals, further research is needed to understand the protective host immune response, new less toxic antifungal drugs, and development of an effective vaccine to prevent coccidioidomycosis. **Figure 2** demonstrates each of the three arsenals discussed in this paper in the battle against *Coccidioides*: host immunity, vaccines, and antifungal drugs. A prophylactic anti-*Coccidioides* vaccine would help to reduce cost associated with long term medical care and frequently needed life-long antifungal drugs. Live attenuated strains have been useful to elucidate our understanding of the protective host immune response against *Coccidioides* which requires T cell mediated immunity, particularly a Th1 and Th17 response. Novel formulations of adjuvants/delivery systems along with immunogenic *Coccidioides* antigens have also been discovered as vaccine candidates. Either could potentially be developed for clinical use. While fluconazole is currently the main antifungal of choice to battle coccidioidomycosis, studies are underway to find less toxic and effective drugs. Altogether, there remains a battle at hand to combat *Coccidioides*, the Dust Devil.

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MV, GT, JG, and BB contributed to the writing, editing, and revision of the manuscript.

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Relevance of Macrophage Extracellular Traps in *C. albicans* Killing

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Candida albicans causes systemic life-threatening infections, particularly in immunocompromised individuals, such as patients in intensive care units, patients undergoing chemotherapy, and post-surgical and neutropenic patients. The proliferation of invading *Candida* cells is mainly limited by the action of the human innate immune system, in which phagocytic cells play a fundamental role. This function is, however, limited in neutropenic patients, who rely mainly on the protective immunity mediated by macrophages. Macrophages have been shown to release extracellular DNA fibers, known as macrophage extracellular traps (METs), which can entrap and kill various microbes by a process called ETosis. In this study, we observed that, upon contact with *C. albicans*, macrophages became active in phagocyting and engulfing yeast cells. ETosis was induced in 6% of macrophages within the first 30 min of contact, and this percentage increased with the multiplicity of infection until a plateau was reached. After 2.5 h incubation, the presence of extracellular macrophage DNA was observed in approximately half of the cells. This study suggests that the formation of METs occurs before pyroptosis (first 6–8 h) and macrophage cell death (up to 24 h), and thus, METs could be included in models describing *C. albicans*–macrophage interactions. We also observed that macrophage ETosis and phagocytosis can occur simultaneously and that, in the first hours of infection, both processes are similarly important in controlling the proliferation of yeast cells, this being critical in neutropenic patients. Finally, it can also be concluded that, since *C. albicans* can degrade DNA, the structural component of METs, yeast extracellular DNase activity can be considered as an important virulence factor.

Keywords: *Candida albicans*, macrophage extracellular traps, multiplicity of infection, antifungal activity, DNase virulence factor

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INTRODUCTION

An effective host response against microbial infections requires the coordinated contribution of the innate and adaptive immune systems (1, 2). Macrophages and neutrophils are essential professional phagocytes that are capable of carrying out various roles in the host's innate defense against pathogens (3, 4), and phagocytosis is an essential stage of this defense (5). This process starts with the engulfment of the hostile cell through actin-dependent mechanisms (6) and formation of the phagosome, which then fuses with the lysosome to produce the phagolysosome (3). Inside the phagolysosome, the pH is lowered, and antimicrobial compounds, reactive oxygen species,

and reactive nitrogen species are produced to induce microbial cell death (7). Hence, phagocytic cells have a key role in the innate immune response and also in the activation of adaptive immune responses.

Recently, a novel antimicrobial process has been described, the extracellular Traps process (ETs). This involves web-like structures composed of double-stranded DNA, histones, antimicrobial peptides, and proteases, which are ejected by immune cells to entrap microbes in a sticky matrix of extracellular chromatin and microbicidal agents (8, 9). These structures were first described in neutrophils and called "neutrophil extracellular traps" (NETs), and the process of killing has been termed "NETosis" (10). Similar extracellular structures were also reported in macrophages (11), mast cells (12), and eosinophils (13), and this mechanism is now generalized as "ETosis." Importantly, the extracellular structures arising from different cell types can exhibit unique features, being distinct from those originally described for neutrophils. The released structures may be composed of chromatin, granule proteins such as neutrophil elastase and myeloperoxidase, and histones (14), and exhibit well-characterized antimicrobial properties (14–16). There are also reports demonstrating that, in addition to chromosomal DNA, mitochondrial DNA could also be used by eosinophils (13) and neutrophils (17) to form ETs. The primary function of ETs has been attributed to their antimicrobial effect. However, the overall role of ETs in host defense remains a topic of debate since the mechanisms behind their formation are still unclear. Besides, recent studies have associated ETs with immunological disorders and pathogenesis of certain vascular physiology disorders, including pre-eclampsia (18) and deep-vein thrombosis (19), as well as autoimmune diseases such as rheumatoid arthritis (20).

Formation of extracellular traps (ETs) generally begins with the loss of nucleus organization followed by chromatin decondensation and nuclear membrane disruption. At the same time, cytoplasmatic granular membranes also undergo disruption and leads to the mixing of granular content with the chromatin leaking into the cytoplasm. Finally, the cellular membrane disrupts, and DNA mixed with the granular content is released into the extracellular milieu (21).

Candida spp. are common pathogens in hospital-acquired infections (22–25), particularly in immunocompromised individuals, among which are intensive care, post-surgical, and neutropenic patients (26). Indeed, *Candida albicans* is the most frequently isolated human fungal pathogen; it causes systemic life-threatening infections, and despite the currently available antifungal therapies, these infections are associated with high mortality and morbidity rates (27, 28).

Candida albicans is known to activate neutrophils to induce NETs development, and these NETs can capture and kill *C. albicans* in both the yeast and hyphal morphologies (15). The released NETs seem to attach to the microbial cell wall, probably through ionic forces, and the protein-containing granules present in the NETs display antimicrobial properties which induce cell death (15). In neutropenic patients, however, the severely reduced neutrophil levels result in reduced antimicrobial effect of NETs. Importantly, *C. albicans* has also been found to induce ET formation in macrophages/monocytes (29, 30) and eosinophils (31), and these may play a protective role in these patients. It has been described that human monocytes release DNA during the initial hours of contact with *C. albicans* and that these ETs have antifungal activity and reduce *C. albicans* growth (29). Murine J774A.1 macrophage-like cells were also found to form ETs, but these were not found to have killing effects on the trapped *C. albicans* (29, 30). In the present study, we show that macrophages exert their antifungicidal activities by phagocytosis and ETosis simultaneously. In our assay, we found that ETosis increases with time and multiplicity of infection (MOI). At a MOI of 25:1, ETosis reached a maximum between 1 and 1.5 h after infection. Interestingly, macrophage cells committed to phagocytosis were not found to undergo ETosis or pyroptosis during the first 4.5 h of interaction. Considering the current model of *C. albicans*–macrophage interaction, these results suggest that METs' formation occurs before pyroptosis (first 6–8 h) and macrophage cell death (up to 24 h). We also observed that the yeast killing efficacy of ETosis and phagocytosis is similar and that *C. albicans* cells can degrade extracellular DNA, which is the main structural component of METs.

MATERIALS AND METHODS

Microbial Strains and Media

Candida albicans clinical isolate SC5314 was used. The strain was stored in 30% glycerol at −80°C and, when needed, maintained at 4°C in yeast extract peptone dextrose (YPD) agar medium containing 1% (*w*/*v*) Bacto Peptone, 0.5% (*w*/*v*) yeast extract, 2% (*w*/*v*) glucose, and 2% (*w*/*v*) agar. For *in vitro* assays, the cells were cultured in YPD medium overnight at 26°C and 140 rpm to maintain cells in the yeast form. Cells were counted in a hemocytometer and normalized to appropriate concentrations. In some cases, dead yeast cells were used, which were prepared by boiling for 30 min.

Macrophages Isolation and Maintenance

Murine macrophage-like cell line J774A.1 was used for most of the experiments. This cell line was maintained at 37°C, in an atmosphere that contained 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Before use, the adherent cells were gently scraped from the plates, centrifuged at 1,200 rpm for 10 min at 4°C, and diluted in 2 ml DMEM. The trypan blue (Sigma-Aldrich) exclusion assay was used for counting and viability analysis, and a suspension of macrophages was prepared at a concentration of 2.5×10^5 cells/ml.

BALB/c bone-marrow-derived macrophages (BMDMs) and macrophages isolated from the peritoneal cavity after eliciting with 8% casein were also used. For the preparation of BMDM, BALB/c mice were killed and their hind limbs removed, isolating the tibia and femur. DMEM medium was injected into the bones and the resulting medium recovered. After centrifugation at 1,200 rpm for 10 min at 4°C, the cell pellet was suspended in Roswell Park Memorial Institute (RPMI)

medium [10 mM HEPES buffer, 0.5 mM 2-β-mercaptoethanol, 50 μg/ml/100 IU/ml streptomycin/penicillin and 10% (*v*/*v*) inactivated fetal bovine serum] supplemented with L929 cell-conditioned medium (LCCM). LCCM was obtained from a fibroblast culture in RPMI for 7 days at 37°C and 5% CO_2, being the source of macrophage-colony-stimulating factor 1. After overnight incubation, the cells were washed by centrifugation at 1,200 rpm for 10 min at 4°C. The cell suspension was then prepared, seeded in 24-well-tissue culture plates, containing coverslips, and incubated for 4 days. Following incubation, 100 μl LCCM was added to each well, and the cells were incubated a further 2 days. Following these 6 days of total incubation time, the medium was renewed and the cells used in the assays as required. For primary murine peritoneal macrophages, mice were injected in the peritoneal cavity with 0.5 ml of an 8% casein solution in PBS. Seventy-two hours after the casein injection, mice were killed by CO_2 exposure and 5 ml of PBS injected for peritoneal lavage. The peritoneal fluids were then carefully recovered, avoiding any possible contamination. Cells were normalized to the desired concentrations and then seeded in tissue culture plates containing coverslips for the assays. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123), the 86/609/EEC directive, and Portuguese rules (DL 129/92).

C. albicans–Macrophage Interaction Assay

The macrophage cell suspensions (1 mL) were seeded into 24-well tissue culture plates, with coverslips in each well, at a cell concentration of 2.5×10^5 cells/mL and incubated overnight at 37°C in 5% CO_2. Macrophages were then infected with 0.2 mL of the *C. albicans* cell suspension at several MOIs: 5:1, 10:1, 25:1, 50:1, and 100:1 (*C. albicans*–macrophages). After 30 min, 1, and 4 h of infection, cells were examined by light microscopy. For Hemacolor staining following incubation, cells were fixed to the coverslips with formol–ethanol (1:9 *v*/*v*) for 1 min, washed with PBS, and stained with Hemacolor Red solution for 3 min followed by Hemacolor Blue solution for min. After staining, the coverslips were washed in water and observed. For fluorescence microscopy, following incubation, cells were fixed with 4% paraformaldehyde, stained with Sytox Green (Molecular Probes) at a final concentration of 5 μM, and observed with a Leica DM5000B fluorescence microscope. A minimum of 250 cells were counted in each slide, from a total of 6 different slides from 3 independent experiments.

MET Induction Through Non-cellular Stimuli: DNA Quantification

Macrophages were seeded overnight at a cell concentration of 2.5×10^4 cells (200 μl) in 96-well-plates before being incubated with lipopolysaccharide (LPS), phorbol-12-myristate-13-acetate (PMA), interferon-gamma (IFN-γ), *N*-acetylglucosamine, and mannan from *Saccharomyces cerevisiae* for 1 h. The concentrations tested ranged from 10 to 1,000 ng/ml LPS, 6.25–200 nM PMA, 6.25–200 ng/ml IFN-γ, 12.5–400 μg/ml *N*-acetylglucosamine, and 31.25–1,000 μg/ml yeast mannan. The cells were then washed with PBS, and Sytox Green (Molecular Probes) was added at a final concentration of 5 μM to detect extracellular DNA. Controls, with macrophages only, were also carried out. The intensity of fluorescence, as a direct quantification of the amount of DNA released, was determined with a Xenogen Vivo Vision IVIS 200 imaging System (Xenogen Corporation, Hopkinton) with excitation at 485 nm and emission at 527 nm. To test double stimuli, macrophages were prepared as described previously and stimulated with LPS at concentrations of 1 μg/ml, 100, and 10 ng/ml for 20 min. The cells were then washed with PBS and incubated with *C. albicans* in PBS at a MOI of 25:1. After 1 h incubation, Sytox Green (Molecular Probes) was added and the amount of DNA released determined as described previously.

Identification of DNA as a Major Structural Component of METs

J774A.1 macrophage-like cells were infected with *C. albicans* at a MOI of 25:1 and incubated at 37°C in 5% CO_2 for 1 h. Cells were then fixed to coverslips, stained with Sytox Green, and treated with the mouse monoclonal antihistone H2A-H2B-DNA complex antibody (gift of Dr. Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin) according to Fuchs et al. (8). The preparations were observed using a Leica DM5000B microscope and Olympus FluoView FV1000 confocal microscope. To further confirm that DNA is the main structural component of METs, macrophages were incubated with *C. albicans*, as described previously, but in the presence of DNase (100 U/ml) and protease (trypsin, 0.25%) at the time of infection. After fixation, cells were stained with Sytox Green or with the Hemacolor staining protocol, as previously described.

Evaluation of *C. albicans* Secreted DNase Activity

J774A.1 macrophage-like cells were incubated with live and heat-killed *C. albicans* cells at a MOI of 25:1 at 37°C in 5% CO_2 for 1 h and the supernatants collected. One arbitrary PCR DNA fragment was incubated with these supernatants for 30 min and then subjected to 1% agarose gel electrophoresis in Tris–borate–EDTA buffer. The gels were stained with ethidium bromide and photographed. Image J software (version 1.51) was used for the analysis of the intensity of DNA fragments in the agarose gel.

Antimicrobial Activity of Macrophage Extracellular Structures

To determine the antimicrobial effect of METs, the survival of *C. albicans* in contact with macrophages was quantified. Macrophages from the cell line J774A.1 were prepared and seeded in 24-well-tissue culture plates as previously described. After adhesion, the medium was removed and replaced with DMEM medium, or with DMEM supplemented with the inhibitor cytochalasin D at a concentration of 0.25 μg/ml. Plates were then reincubated for 20 min and the medium removed before adding the *C. albicans* suspension simultaneously with treatment with DNase (100 U/ml). The cells were further incubated at 37°C, 5% CO_2 for 1 h and 800 μl water with 10%

saponin added to each well to facilitate removal of adhered *C. albicans* cells from the wells. To confirm cell removal, up and down pipetting was performed several times, and the plates were examined under a binocular magnifier. The obtained suspension was diluted and seeded on YPD agar plates and incubated overnight at 37°C before counting to determine the number of viable *C. albicans* cells as colony-forming units.

Statistical Analyses

Statistical analyses were performed using Graph Pad Prism (version 7), and significance was determined using two-way ANOVA with Tukey's multiple comparison test. All tests were performed with a confidence level of 95%. All experiments were done in duplicate, and results were obtained from three independent experimental assays.

RESULTS

Macrophage Extracellular Fiber Production in Response to *C. albicans* Cells

Macrophages are dynamic cells, distributed in various tissues, which play important roles in immune processes and whose functions are vital to host immune defense and tissue homeostasis. These professional phagocytes play key roles in initiating inflammation and orchestrating its resolution. Their main conventional antimicrobial ability is to eliminate

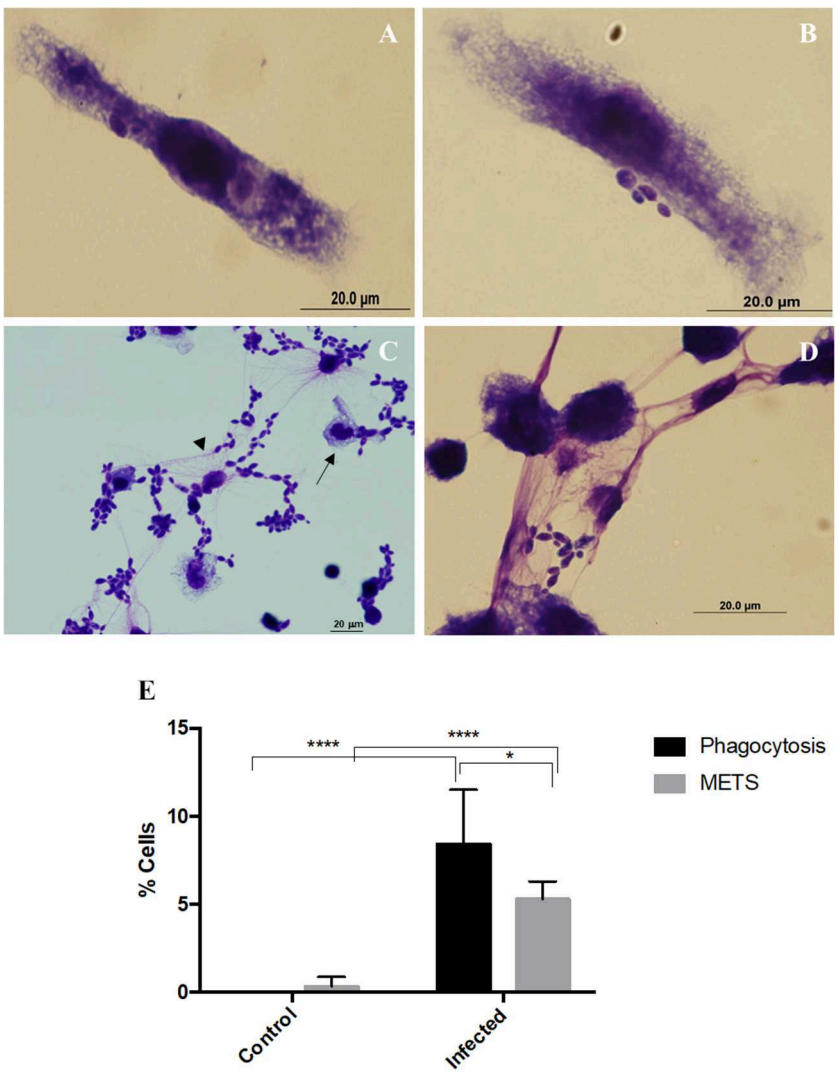

FIGURE 1 | Extracellular fibers observed after macrophages–*C. albicans* interaction *in vitro*. Hemacolor staining images of the structures observed following *in vitro* interaction of *C. albicans* and J774A.1 macrophage-like cells for 30 min. Macrophages exhibiting typical phagocytic structures **(A,B)**. Extracellular structures resembling macrophage extracellular traps (METs) **(C,D)**. The arrowhead points to extracellular structures that entrap *C. albicans* cells and the arrow to phagocytosis. Quantification of macrophages exhibiting phagocytosis and METs **(E)** after infection with *C. albicans*. $^{*}P < 0.05$ and $^{****}P < 0.0001$ by the Tukey's multiple comparisons test.

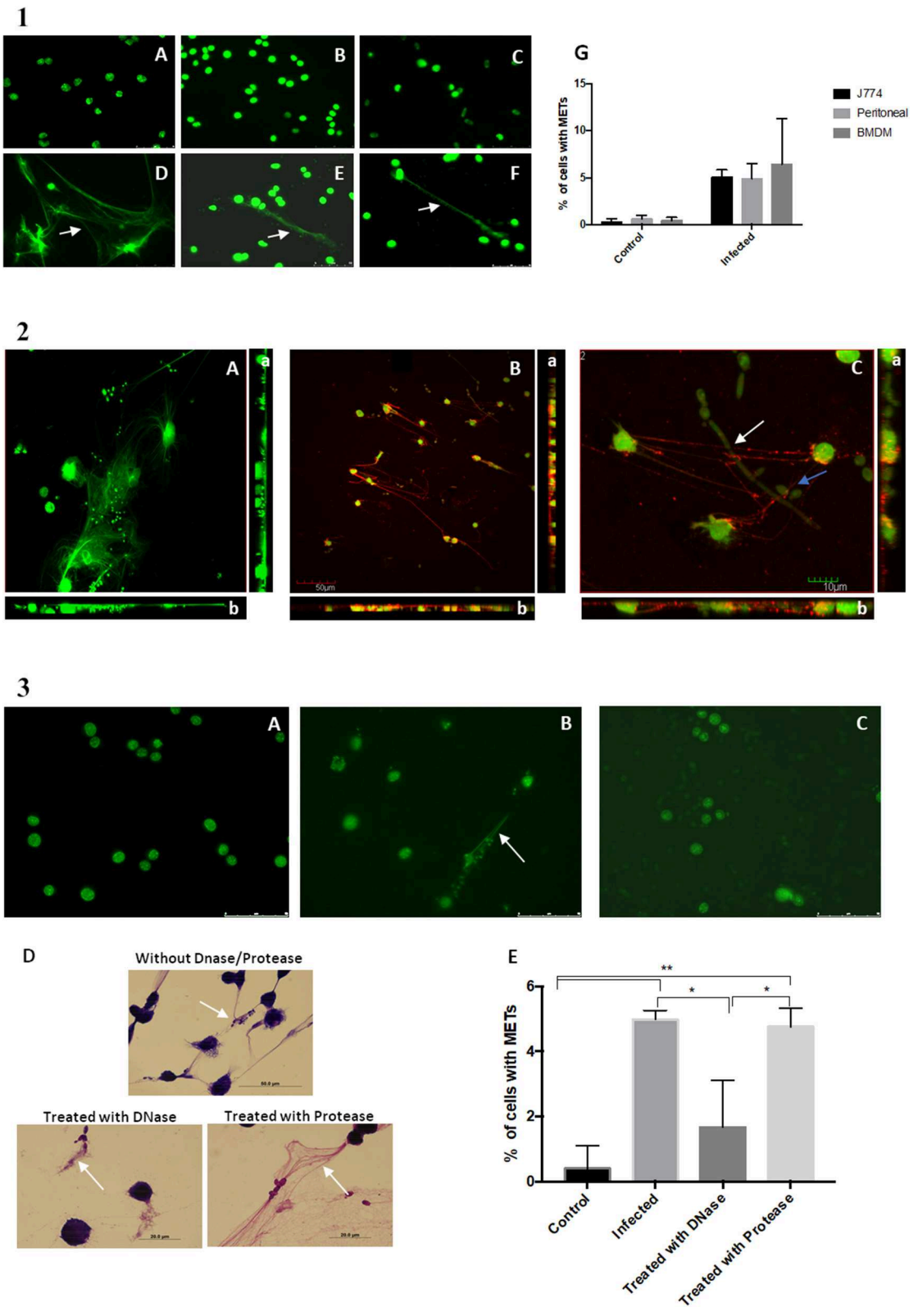

FIGURE 2 | DNA release following *in vitro* incubation of macrophages with *C. albicans* cells. **(1)** Fluorescence images of macrophage cells incubated with *C. albicans* for 30 min and stained with Sytox Green. Control of J774A.1 macrophage-like cells (A) and J774A.1 macrophage-like cells incubated with *C. albicans* (D). Control

(Continued)

FIGURE 2 | cells of primary murine peritoneal macrophages (B) and the same cells incubated with *C. albicans* (E). Bone-marrow-derived macrophages (BMDM) from BALB/c mice control cells (C) and BMDM infected with *C. albicans* (F). Quantification of macrophages exhibiting macrophage extracellular traps (METs) (G) after infection with *C. albicans*. **(2)** Fluorescence images of J774A.1 macrophage-like cells entrapping *C. albicans* stained with Sytox Green and anti-H2A-H2B-DNA complex antibody (B); macrophages in more detail entrapping *C. albicans* yeast cells (blue arrow) and hyphae (white arrow) (C); J774A.1 macrophage-like cells incubated with *C. albicans* cells and stained only with the secondary antibody and Sytox Green (A). Rotation around the *y*-axis (a); rotation around the *x*-axis (b). Red corresponds to the labeling with anti-H2A-H2B-DNA complex antibody; green corresponds to the marking with Sytox Green. **(3)** Fluorescence images of control J774A.1 macrophage-like cells (A), macrophages infected with *C. albicans* (B), and macrophages infected with *C. albicans* and treated with DNase (C) followed by Sytox Green staining. Hemacolor images (D) of macrophages infected with *C. albicans* and treated with trypsin, with DNase, or not treated. Quantification of macrophages exhibiting METs (E) after infection with *C. albicans* in the presence and absence of treatments. $^{*}P < 0.05$ and $^{**}P < 0.01$ by the Tukey's multiple comparisons test.

microbial pathogens through phagocytosis, using a combination of oxidative and non-oxidative microbicidal mechanisms.

Incubation of macrophages with *C. albicans* was performed and cells fixed and stained with Hemacolor (**Figure 1**). Different structures were observed in this assay, namely elongated macrophages with yeast cells attached or internalized (**Figures 1A**,**B**), and macrophages interconnected to each other by extracellular structures that resembled ETs trapping the yeast cells (**Figures 1C**,**D**). After 30 min incubation with *C. albicans*, 8.4% of macrophages were engaged in phagocytosis and 5.3% formed extracellular structures (**Figure 1E**). In a more detailed analysis, it was possible to observe that both types of morphologies were present simultaneously (**Figure 1C**, arrows).

Macrophage Release of Extracellular DNA

To determine whether these extracellular structures consisted of DNA, the assay was repeated with not only the macrophage-like cells from J774A.1 but also BMDM and primary murine peritoneal macrophages. At the end of the incubation period, the cells were fixed with 4% paraformaldehyde, stained with Sytox Green and observed under a fluorescence microscope (**Figure 2.1**A–F). A quantification of cells with METs was also performed (**Figure 2.1**G). Results show that the extracellular structures released by J774A.1 macrophage-like cells (**Figure 2.1**D), by a derived primary culture (BMDM) (**Figure 2.1**F), and by primary murine peritoneal macrophages (**Figure 2.1**E) consisted of DNA, since they were stained by the DNA staining dye Sytox Green. As expected, uninfected macrophages showed no or only residual ET structures (**Figure 2.1**A–C).

Confocal microscopy confirmed that these ETs could be readily visualized with an antibody against histone–DNA complexes, which allowed us to confirm that DNA and histones are the main components of these structures (**Figure 2.2**). These results are in agreement with the previous observed phenotypes of NETs (14). In addition, it was possible to observe that the METs could entrap both yeast and hyphae *C. albicans* structures (**Figure 2.2**C and **Videos S1**, **S2**).

Induction of the METs was performed in the presence of DNase and protease (trypsin) and resulted in a significant difference in the percentage of ETs being produced (**Figure 2.3**E). Fluorescent (**Figure 2.3**C) and Hemacolor images (**Figure 2.3**D) showed no clear formation or destruction of METs in the presence of DNase, in contrast to induction of ETs in the absence of DNase (**Figure 2.3**B) or the presence of protease. These results confirmed that the ETs contain DNA and are thus susceptible to DNase degradation.

ET Formation Increases With Increasing Multiplicity of Infection

Incubation of macrophages with *C. albicans* at different MOIs of 5:1, 10:1, 25:1, 50:1, and 100:1 (*C. albicans*–macrophages) showed that increasing the number of *C. albicans* cells induced an increase in the percentage of METs produced (**Figure 3**) until an MOI of 25:1. Above an MOI of 25:1, no significant differences were observed in the frequency of ETs, regardless of the number of yeast cells present.

Macrophages Involved in Phagocytosis Do Not Undergo ETosis

The kinetics of MET formation was evaluated in primary murine peritoneal macrophages after infection with *C. albicans* cells and evaluated over 2.5 h by real-time imaging in the presence of Sytox Green (**Video S3**). **Figure 4.1**A shows representative images taken at different time points. In **Figure 4.1**B, we can observe that the percentage MET production increased progressively with time. Statistical analysis indicated that nuclear staining was significantly different from baseline after only 78 min incubation (30.7% METs) but that between 87 min (34.8% METs) and 150 min (56.0% METs), there were no significant differences. This analysis also showed that MET morphology is not uniform (**Figure 4.2**A); cells may present a diffuse extracellular chromatin morphology (puffball-like) (**Figure 4.2**A, asterisk), a spread extracellular chromatin morphology (comet-like) (**Figure 4.2**A, number sign), or an extended strands morphology that interlinks across cells (**Figure 1**). These morphologies were already described in other studies (32).

The kinetics of the formation of METs is also variable. Some cells were found to present a puffball-like morphology (**Figure 4.2**A, dashed arrow) and remained as such during the 2.5-h analysis time, while others were Sytox Green positive and progressed rapidly (16 min total) to a comet-like morphology (**Figure 4.2**A, solid arrow). Others still, as represented by the cell in **Figure 4.2**B, were already in a diffuse morphology at the beginning of the video analysis but progressed to a comet-like morphology after 2 h and 8 min.

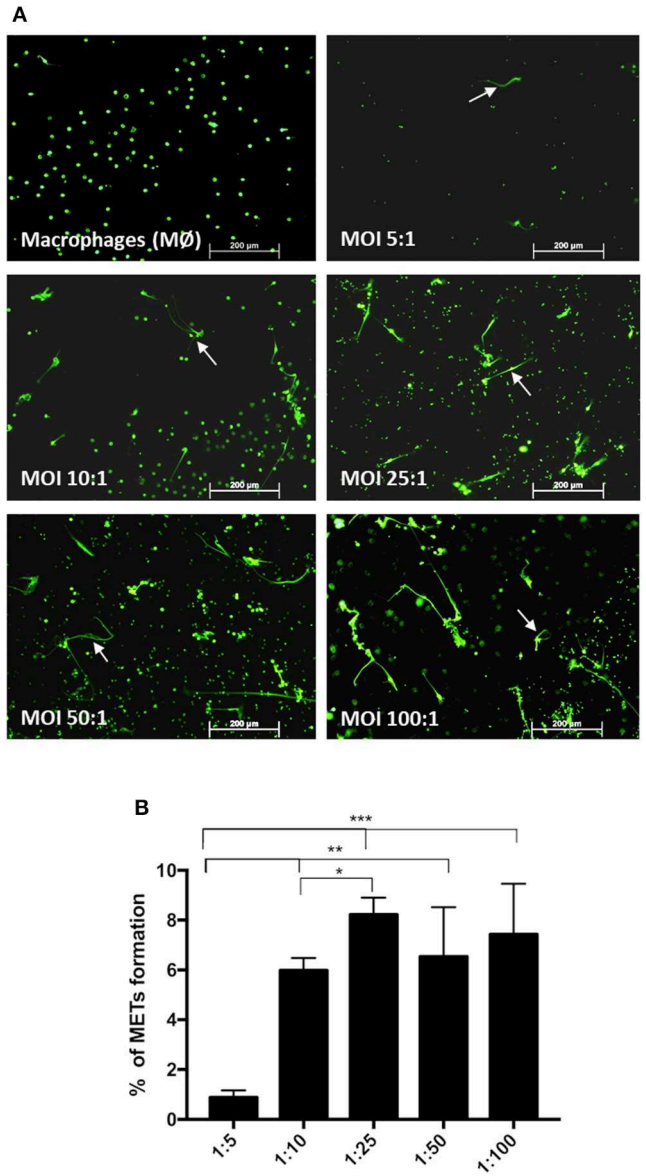

FIGURE 3 | Frequency of macrophage extracellular trap (MET) formation with multiplicity of infection. Fluorescence images (arrows point to METs) **(A)** and quantification of the formation of METs **(B)** by J774A.1 macrophage-like cells incubated with *C. albicans* at different MOI (5:1, 10:1, 25:1, 50:1, and 100:1; *C. albicans*–macrophages) for 30 min and stained with Sytox Green. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by the Tukey's multiple comparisons test.

Candida albicans cells can form filaments inside macrophages that could disrupt the macrophage cell, and thus, the Sytox Green signal observed could be due to this disruption and not to MET release. Therefore, a closer look was taken at cells undergoing phagocytosis overtime (**Figure 4.3** and **Video S4**). Results show that *C. albicans* cells are efficiently phagocytosed by macrophages; however, once phagocytosed and contained within a phagosome, the yeast can still form hyphae, leading to the stretching of phagocyte membranes. Nevertheless, during the analysis of 4.5-h duration, these macrophages did not stain with Sytox Green, which indicates that they did not induce METs or undergo pyroptosis. Considering the current model of *C. albicans*–macrophage interaction, these results suggest that MET formation is a mechanism that occurs before pyroptosis (first 6–8 h) and macrophage cell death (up to 24 h).

Induction of METs by Non-cellular Stimuli

The yeast cell wall is composed of an outer layer of glycosylated proteins with mannosyl residues and an inner layer of β-glucans and chitin (33). Taking into account that *C. albicans* induced the formation of METs, we tested whether, individually, mannans (mannose polymer) or *N*-acetylglucosamine (the monomeric unit of chitin) could induce the formation of METs. In addition, as PMA and LPS stimuli have been shown to activate neutrophils to release NETs (14), and as IFN-γ is an important cytokine in macrophage activation, we also tested these stimuli in macrophages. The results showed that, except for LPS, the tested stimuli did not significantly enhance the formation of METs, and LPS only induced a slight increase after 1-h incubation (**Figure 5**). Therefore, we decided to quantify the formation of METs when macrophages were incubated with LPS and *C. albicans* (**Figure 5**). Results indicate significant differences ($P < 0.05$) in the formation of METs for macrophages incubated with *C. albicans* as compared with macrophages alone. When *C. albicans* and LPS were combined, a significant difference was also observed in comparison with macrophages alone: *C. albicans* with 1 µg/ml LPS $P < 0.0001$ and *C. albicans* with 100 ng/ml LPS $P < 0.05$. However, since no significant differences were observed with LPS incubations alone, these differences with *C. albicans* and LPS were mainly due to the presence of the yeast. Thus, even combining *C. albicans* with LPS, the release of METs is mainly due to the presence of *C. albicans* and not to LPS.

Dead and Live *C. albicans* Cells Are Able to Induce METs

Since the different components of the *C. albicans* cell wall were not able to significantly induce the formation of METs, we incubated macrophages with *C. albicans* live and heat-killed cells at the same MOI (25:1) for 1 h (**Figure 6**). Results indicate that dead *C. albicans* cells are also able to induce macrophage extracellular structures (**Figure 6A**) and even showed a higher amount of these structures (10.9% ± 2.5) when compared with macrophages incubated with live yeast cells (5.2% ± 1.4) (**Figure 6B**). One possible explanation for this difference is that the live *C. albicans* cells can secrete compounds, such as DNases, which may degrade DNA, the main component of METs. To determine if *C. albicans* secretes compounds with the ability to degrade DNA, an arbitrary DNA fragment was incubated with the supernatant of macrophages infected with live and heat-killed *C. albicans* cells (**Figure 6C**). Agarose gel electrophoresis analysis indicated ∼25% degradation of the DNA fragment upon incubation with the supernatant of macrophages stimulated with live *C. albicans* cells (**Figure 6D**). In contrast, the DNA fragment incubated with the supernatant of macrophages stimulated with dead *C. albicans* cells and with the control (uninfected macrophages) was unaltered. These results suggest

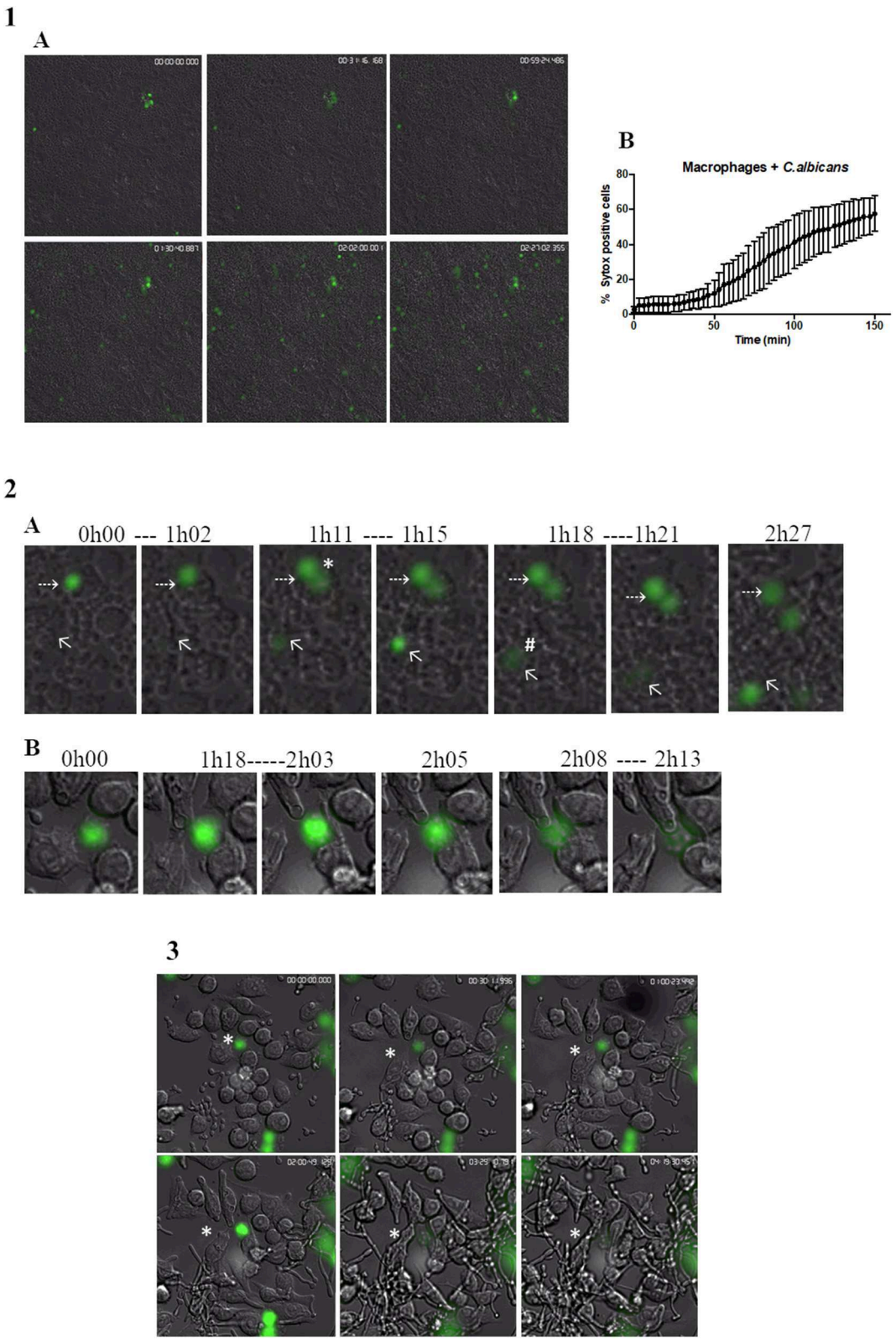

FIGURE 4 | Live cell imaging of macrophages incubated with *C. albicans*. (**1**A) Representative images of primary murine peritoneal macrophages incubated with *C. albicans* and Sytox Green, images taken at selected time points during the 2.5-h incubation time (images taken with a 10× objective). (**1**B) Quantification of

(Continued)

FIGURE 4 | Sytox-positive cells over time. Images representative of nine replicates. (**2**A) Representative images of macrophage cells observed in (**1**A) where we can discern different MET morphologies. Asterisk represents a diffused extracellular chromatin and number sign a spread extracellular chromatin. (**2**B) Representative images of the kinetics of MET formation (images from **3**). Dashed arrows points to a cell that remained at the puffball-like morphology over the time studied. Solid arrows points to a cell that progressed to comet-like morphology. **(3)** Representative images of primary murine peritoneal macrophages incubated with *C. albicans* and Sytox Green, images taken at selected time points during the 4.5-h incubation time (images taken with a 40× objective). Images representative of six replicates.

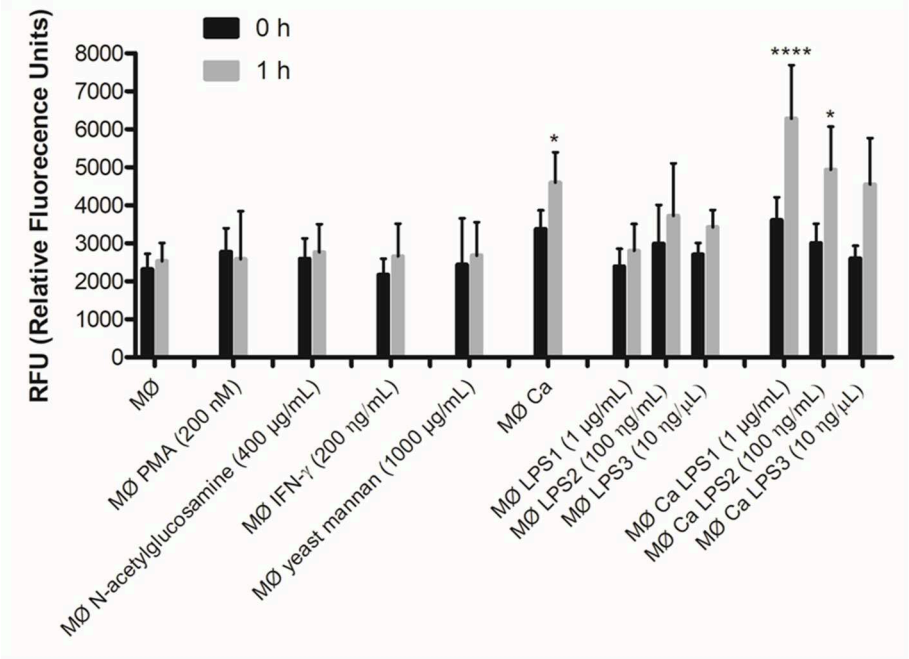

FIGURE 5 | DNA release in response to non-cellular stimuli and *C. albicans*. J774A.1 macrophage-like cells stimulated with 200 nM phorbol-12-myristate-13-acetate (PMA), 400 µg/ml *N*-acetylglucosamine, 200 ng/ml interferon-gamma (IFN-γ), 1,000 µg/ml yeast mannan, 1 µg/ml [lipopolysaccharide 1 (LPS1)], 100 ng/ml (LPS2), or 10 ng/ml (LPS3) LPS, and incubated with *C. albicans* alone or with yeast and LPS simultaneously. Extracellular DNA was quantified by fluorescence analysis. $^{*}P < 0.05$ and $^{****}P < 0.0001$ by the Tukey's multiple comparisons test, in comparison with macrophages alone.

that *C. albicans* cells can secret compounds that can degrade the ETs and thereby lead to the observed lower MET levels.

Macrophage Extracellular Structures Trap and Kill *C. albicans* Cells

To test if the extracellular structures can kill the entrapped *C. albicans* cells, we infected J774A.1 macrophage-like cells with the yeast and quantified the percent survival by CFU counting. The percentage of *C. albicans* survival was determined either in the presence of cytochalasin D, which inhibits intracellular death, or in the presence of DNase I, inhibiting extracellular death (**Figure 7**). In addition, samples of macrophages incubated with *C. albicans* in the presence of both cytochalasin D and DNase I were used as the 100% survival control (34).

After 1-h incubation with macrophages, only 48.88% ± 12.8 of the *C. albicans* cells survived macrophage antimicrobial action. However, with the administration of DNase, the survival percentage increased to 64.96 ± 11.8 ($P = 0.0242$), which indicates that METs contribute to *C. albicans* killing. The same observation was made following cytochalasin D treatment; here, the survival was 74.10% ± 11.5, being significantly different to macrophages incubated with *C. albicans* without any treatment ($P = 0.0005$) and demonstrating that phagocytosis, as expected, is important in the battle against *C. albicans*. Furthermore, although the difference in the survival percentage between the two different treatments is ∼10%, this difference is not statistically significant and suggests that both phagocytosis and METs are similarly important in *C. albicans* killing. As a control, *C. albicans* growth in the presence of cytochalasin D and DNase was also evaluated in the same conditions, and a viability of about 93% ± 1.4 was observed, confirming that these treatments do not affect *C. albicans* growth. Thus, it can be concluded from these experiments that METs have an antimicrobial efficacy similar to that of phagocytosis.

DISCUSSION

Human fungal pathogens causing invasive infections are responsible for around one and a half million deaths every year (35). *Candida* spp. are common pathogens and are the fourth most frequent cause of nosocomial bloodstream infections, with *C. albicans* being particularly common. This human opportunistic pathogenic fungus often causes systemic life-threatening infections in immunocompromised individuals. Despite currently available antifungal therapies, *C. albicans*-associated mortality and morbidity remain high,

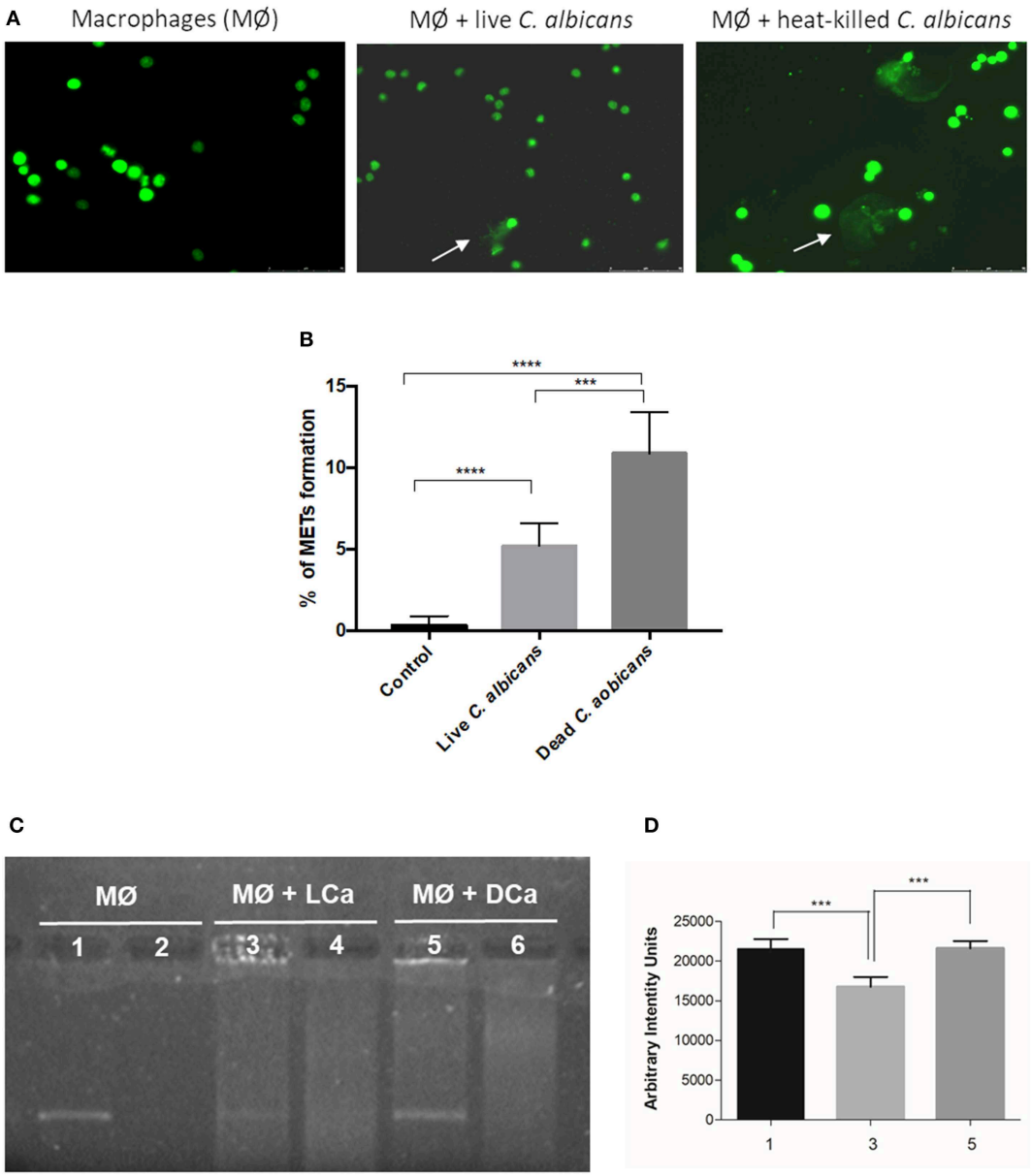

FIGURE 6 | MET formation by live and heat-killed *C. albicans* cells. Representative fluorescence images of J774A.1 macrophage-like cells infected with live cells and heat-killed *C. albicans* **(A)** and quantification of macrophage extracellular trap (MET) formation **(B)**. Arrows indicate the presence of macrophage extracellular traps (ETs). Evaluation of DNase activity in the supernatants of incubations: agarose gel **(C)** and intensity quantification **(D)** of a DNA fragment incubated with supernatant of macrophages alone (1), supernatant of macrophages infected with live *C. albicans* cells (3), and supernatant of macrophages infected with heat-killed *C. albicans* cells (5). Negative controls, without DNA addition, of (2) only the supernatant of macrophages, (4) the supernatant of macrophages infected with live *C. albicans,* and (6) the supernatant of macrophages infected with heat-killed *C. albicans*. ***$P < 0.001$ and ****$P < 0.0001$ by the Tukey's multiple comparisons test.

and more than 50% of infected patients die due to systemic candidemia (27, 28).

Invading *Candida* cells are immediately attacked by the human innate immune system, which involves activation of the complement system, generation of antimicrobial peptides, and action of phagocytic immune cells. The important contribution of phagocytes to the innate immune control of infections is based on phagocytosis, but more recently, ETosis has also been identified. Several studies have indicated that the formation of ETs is an effective mechanism in combating invading pathogens (36). Macrophage phagocytes play key roles in host immune defense and tissue homeostasis, and, in addition to its phagocytic classical function, recent investigations have shown that macrophages are capable of also producing extracellular traps (ETs) that contribute to their antimicrobial function

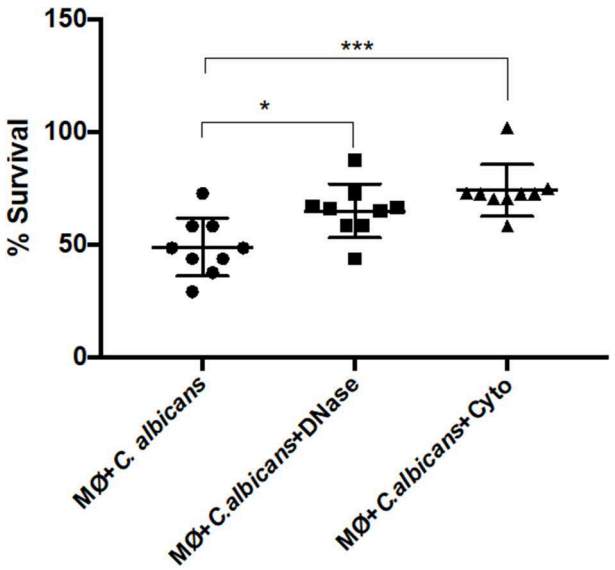

FIGURE 7 | *C. albicans* killing by macrophage extracellular traps (METs). *C. albicans* survival (%) when incubated with J774A.1 macrophage-like cells without any treatment, or with treatment with DNase and/or cytochalasin D. Samples treated with cytochalasin D and DNase I simultaneously were set as the 100% survival control group. $^{*}P < 0.05$ and $^{***}P < 0.001$ by the Tukey's multiple comparisons test.

(8, 9, 11). Nevertheless, in macrophages, this contribution is still poorly understood.

In this study, we observed that *C. albicans* induce macrophage ETs formation in murine J774A.1 macrophage-like cells, peritoneal macrophages, and BMDM. Morphology analysis showed macrophages interconnected with each other, trapping the yeast cells within extracellular structures. These interconnected fibrous structures, which were formed between several macrophages and *Candida* cells, have also been described for human monocytes, forming cluster-like structures where groups of yeast cells are interlaced (29). In our assay, the frequency of cells undergoing ETosis ranged from 6% after 30 min incubation to approximately half of the cells after 2.5 h. The values observed are within the ranges reported in the literature, which ranged from ∼10 to 50% depending on the time of incubation (11, 29, 30, 32, 37, 38). Characterization of these extracellular structures, staining with Sytox Green, incubation with anti-H2A-H2B-DNA complex antibody, and treatment with DNase confirmed that their main structural component is DNA, as expected (36). Treatment with DNase simultaneous to *C. albicans* incubation did not prevent formation of METs, but the extracellular structures appeared degraded, and the percentage of cells with clear MET formation was reduced. This is in agreement with previously reported observations for bovine macrophages that indicated that METs are susceptible to DNase degradation (11, 29, 30, 37). In the present study, we also discerned that increasing the MOI increased the amount of METs observed, but only until a MOI of 25:1, after which the formation of METs did not increase, regardless of the number of yeast cells present. ETs have been proposed to restrict microbes to the site of infection, thus preventing systemic spreading, which is particularly important for controlling invasive infections by fungal pathogens (39, 40). Since macrophages are tissue cells, being one of the first innate immune cells to encounter invading pathogens, the intertwisted METs, interconnected with each other and trapping the yeast cells, will play an important role in controlling the spread of *C. albicans* cells.

Real-time imaging revealed that, upon contact with *C. albicans*, macrophages rapidly engulfed yeast cells. The percentage of Sytox Green positive cells increased with time, reaching a significant difference from baseline after 78 min incubation, with 30.7% positive cells. Thereafter, the number of Sytox Green positive cells continued to increase until 150 min incubation, reaching 56.0%. The pattern of MET formation was observed to be non-uniform; we observed cells that presented a puffball-like morphology, while other cells presented a comet-like morphology, remaining as such for 2.5 h. We also observed cells that initially showed a puffball-like morphology but later presented a comet-like morphology. These results are in agreement with those previously reported for vertebrate (41) and invertebrate phagocytes (32). In addition, the time frame of MET formation is also in agreement with that previously described for human monocytes; that being that, upon microbial contact, DNA was released within 2–4 h, but in a few cases, the release of extracellular DNA was detected even earlier, after 20–40 min (29).

In this study, during the real-time analysis, macrophages involved in phagocytosis of *C. albicans* cells did not stain with Sytox Green even after 4.5 h incubation. It is well-known that, once phagocytosed, *C. albicans* cells contained within the phagosome can form hyphae, which can lead to stretching of the phagocyte membranes and eventually membrane piercing and killing of the macrophages (42, 43). These studies show that macrophage killing by *C. albicans* occurs in two distinct phases: phase 1 (first 6–8 h) and phase 2 (8–10 h to 18–24 h post-phagocytosis). The early phase is associated with pyroptosis, a proinflammatory macrophage death, whereas the latter phase depends on robust hyphal formation and is independent of pyroptosis. Our study was performed within the first 4.5 h of *C. albicans* interaction with phagocytes and thus was before the time frame described for pyroptosis. Since macrophages involved in phagocytosis did not become Sytox Green positive during the analysis, DNA release by pyroptosis may not yet be relevant in these initial hours of interaction. This result is in agreement with studies of human monocyte ETs, which indicated that ETosis was an early coordinated process occurring upon contact with *C. albicans*, and different from pyroptosis (29).

In contrast to NETs, METs are not induced by LPS or PMA, not even when LPS was incubated with macrophages previous to *C. albicans*, which indicates that no synergism occurs in MET formation. In addition, neither mannans nor *N*-acetylglucosamine, two components of the yeast cell wall, were able to induce the formation of METs. However, as reported for NETs, heat-killed yeast cells were able to induce METs and in higher amounts than live yeast cells. This suggests that METs, in contrast with NETs, may be induced specifically by microbial cells, alive or dead, rather than by microbial components.

Microorganisms are known to have several strategies to evade human immune attack (44). The most prominent *C. albicans* evasion mechanism is the morphological switch to hyphal growth, which can pierce through human phagocyte membranes, killing the cell (42). However, it has also been described that different *C. albicans* isolates can present extracellular DNase activity (45). We observed that the live yeast cells that we used secreted components with DNase activity that degraded an arbitrary DNA fragment. This is in agreement with that observed for NETs, where *C. albicans* cells were able to degrade DNA in the NETs, particularly strain SC5314, the same strain used in our study (46). Since this extracellular DNase activity is strain dependent, this activity may be considered a virulence factor facilitating yeast escape from ET killing by degradation of ETs.

Previous studies have shown that ETs from neutrophils, eosinophils, and mast cells kill microbes, including *Candida* cells (14–16). In this study, we observed that after 1 h incubation, macrophages were able to kill ∼50% of the yeast cells, but when DNase was added, only 35% of the yeast cells were killed, indicating that METs contribute to *C. albicans* killing. However, in the presence of cytochalasin D, the percentage of yeast killing was just 26%, confirming the importance of phagocytosis in killing *C. albicans.* Although there are differences in the percentages of yeast killing by the different treatments, results indicate that both phagocytosis and ETosis play a role in the *C. albicans* killing by macrophages. This result is similar to that reported for METs, which showed that human monocytes have antifungal activity (29), but contrary to that reported by Liu et al. (30) for macrophages. This difference may be due to the multiplicity of infection used by Liu et al., which, according to our results, induces only low amounts of METs.

In conclusion, our study demonstrates that macrophages form ETs when exposed to *C. albicans*, and these can entrap and kill the fungal cells. METs seem to be induced by whole *Candida* cells as opposed to by their cell wall components, and moreover, live yeast cells have the capacity to counterattack METs by degrading its main structural component, DNA. Considering the current model of *C. albicans*–macrophage interaction, it seems that the formation METs is a mechanism that occurs prior to pyroptosis (first 6–8 h) and macrophage cell death (up to 24 h) and could be included in this model.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AL performed the experiments, analyzed, interpreted data, and wrote the manuscript. CP supervised the experiments and interpreted data. PS supervised the experiments, analyzed, interpreted data, and assisted in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02767/full#supplementary-material

Video S1 | 3D Animation-x rotation of immunofluorescence microscopy.

Video S2 | 3D Animation-y rotation of immunofluorescence microscopy.

Video S3 | Real-time imaging of *C. albicans*/macrophage interaction (amplification 100×).

Video S4 | Real-time imaging of *C. albicans*/macrophage interaction (amplification 400×).

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LncSSBP1 Functions as a Negative Regulator of IL-6 Through Interaction With hnRNPK in Bronchial Epithelial Cells Infected With *Talaromyces marneffei*

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Talaromyces marneffei (TM) is an important opportunistic pathogenic fungus capable of causing disseminated lethal infection. In our previous study, we identified host lncRNAs and mRNAs that are dysregulated in TM-infected bronchial epithelial cells. In this report, we verified that IL-6, a key factor in acute inflammatory response, is down-regulated in TM pathogenesis. To elucidate the mechanism of IL-6 regulation, we analyzed the coding/non-coding network, and identified lncSSBP1, a novel lncRNA that is up-regulated by TM. Our results demonstrate that overexpression of lncSSBP1 decreases IL-6 mRNA expression, whereas knockdown of lncSSBP1 enhances IL-6 mRNA expression. Though lncSSBP1 is primarily localized to the nucleus, bioinformatics analysis suggests that it is unlikely to function as competing endogenous RNA or to interact with IL-6 transcription factors. Instead, RNA pull down and RNA immunoprecipitation assays showed that lncSSBP1 binds specifically to heterogenous nuclear ribonucleoprotein K (hnRNPK), which is involved in IL-6 mRNA processing. Our findings suggest that lncSSBP1 may affect IL-6 mRNA expression during TM infection through interaction with hnRNPk in bronchial epithelial cells. Our results suggest a novel pathway by which TM may suppress the immune response to its advantage.

Keywords: microbial immunology, fungus, *Talaromyces marneffei*, lncRNA, IL-6, hnRNPK, bronchial epithelial cell

INTRODUCTION

Talaromyces marneffei (TM), formerly known as *Penicillium marneffei*, is a prominent opportunistic pathogen capable of causing disseminated lethal infection. This thermal dimorphic pathogenic fungus infects immunocompromised hosts including those with acquired immunodeficiency syndrome (AIDS) (1). Disseminated TM is mainly endemic to southern China, Hong Kong, Taiwan and in southeastern Asia (2). In mainland China, the majority of TM infection cases are reported in southern China, and more than 80% of them are from Guangdong and Guangxi Provinces (3). With increased immunodeficiency and immunocompromise, the incidence

of TM infection has been steadily increasing, leading to ∼50,000 new cases of TM infections diagnosed each year (4). In endemic areas, TM together with tuberculosis and cryptococcosis, are the top three opportunistic infections in AIDS patients (5). Current predictions suggest that 16.1% of AIDS patients in Guangxi Province suffer from TM infection, and the mortality rate of TM infection is significantly higher than that of any other AIDS complication (6). If appropriate systemic antifungal therapy is not administered in a timely fashion, the mortality rate can reach as high as 33% (7). Furthermore, the recurrence rate of disseminated TM is up to 50% (8).

In the course of a typical TM infection, TM conidia are inhaled into the lower respiratory tract, adhere and penetrate to bronchial epithelial cells, are phagocytized by alveolar macrophages, and lurk in the reticuloendothelial system (9). The conidia can disseminate around the body through the reticuloendothelial system (10). Timely initiation and activation of the innate immune response are essential for the host to clear the pathogen. As an important part of the innate immune system, bronchial epithelial cells play a key role in preventing pathogen invasion and inducing subsequent immune responses and immune evasion (11). Therefore, increased understanding of the response of epithelial cells to TM provides a key strategy for developing novel therapies to combat TM pathology and spread.

IL-6, a critical pleiotropic molecule in inflammation, imparts context-dependent pro-inflammatory and anti-inflammatory properties (12). Binding of IL-6 to the IL-6 receptor mediates intracellular signal transduction pathways, which often serve to boost innate immune responses during pathogenic infections (13). Recent studies have shown that IL-6 activates the classic signaling pathway via membrane bound IL-6 receptors and the trans-signaling pathway via soluble IL-6 receptors (12–14). Classic signaling is mainly considered to be protective and regenerative (anti-inflammatory), while trans-signaling is considered pro-inflammatory. Therefore, IL-6 is an important regulator of the balance between pro-inflammatory and anti-inflammatory responses that may be modulated by pathogens to temper the host's response.

In our previous study, to reveal the relationships between TM and lncRNAs, we used microarray to profile dysregulated lncRNAs and mRNAs in a human bronchial epithelial cell model that was infected with TM conidia for 4 h, including IL-6, which was down-regulated by TM (15). In this report, we have extended these findings by demonstrating that lncSSBP1 negatively regulates IL-6 expression in TM-infected bronchial epithelial cells. Furthermore, we demonstrate that heterogenous nuclear ribonucleoprotein K (hnRNPK) serves as a binding partner of both lncSSBP1 and IL-6 mRNA. Therefore, our results elucidate a pathway by which lncSSBP1 may regulate innate immunity in TM-infected cells.

MATERIALS AND METHODS

Compliance With Ethical Standards

The TM strain was isolated from the sputum an HIV-negative patient suffering from disseminated TM infection at the first affiliated hospital of Guangxi Medical University, as described in our previous study (15, 16). The isolation was a part of standard care of the patient. Subsequent isolation of the microorganism was undertaken according to standard laboratory processes. Our study was approved by the First Affiliated Hospital of Guangxi Medical University Ethical Review Committee.

TM Conidia Preparation

The strain was cultured on potato dextrose agar medium (Luqiao Technology, Beijing, China) at 25°C for 7–10 days. Colonies were washed with sterile phosphate buffed saline, and then conidia were collected by centrifugation.

Cell Culture and Infection

The human bronchial epithelial cell line BEAS-2B was preserved in our laboratory, Guangxi Medical University, Guangxi, China. Cells were cultivated in RPMI1640 medium mixed with 10% fetal bovine serum (Invitrogen, Carlsbad, USA), and were placed in cell incubator at 37°C with 5% CO_2. To initiate infection, BEAS-2B cells were stimulated with TM conidia for 4 h.

Transmission Electron Microscopy

Cells were fixed with 1% glutaraldehyde and 4% paraformaldehyde in PBS at 4°C overnight followed by post-fixation in 1% buffered osmium tetroxide. Cells were dehydrated through a graded series of ethanol (30, 50, 70, 80, 90, and 100%) for 10 min each. The cells were then embedded in epoxy resin and polymerized. After polymerization, the embedded cells were cut into ultrathin sections using an ultramicrotome (Leica, Wetzlar, Germany), and stained with 1% uranyl acetate followed by lead citrate. All samples were viewed on a transmission electron microscope (Hitachi H-7650, Tokyo, Japan).

RNA Extraction and qRT-PCR

Total RNA was extracted from cells from both the control and treatment groups with TRIzol reagent (Invitrogen, Carlsbad, USA), following the manufacturer's instructions. The RNA quality and quantity were measured with a Nucleic Acid Protein Detector (Thermo Fisher Scientific, Waltham, USA). Total RNA was used to transcribe into cDNA with the First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). PCR reactions were run using the following profile: 1 cycle at 95°C for 30 s; 40 cycles of 95°C for 3 s and at 60°C for 30 s. The primers used for qRT-PCR are listed in **Table S1**. GAPDH expression served as the internal control, and the relative expression levels of lncRNA and mRNA were determined using the $2^{-\Delta\Delta Ct}$ analysis method (17).

Measurement of IL-6

Levels of IL-6 in cell supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (Cat #CSB_E04638h; CUSABIO, Wuhan, China) according to the manufacturer's instructions.

Construction of a Coding/Non-coding Gene Co-expression Network

A coding/non-coding co-expression network (CNC network) was constructed based on our existing microarray data (15). The Pearson correlation coefficient (PCC) between IL-6 and differentially expressed lncRNAs was calculated with |PCC|

$\geq$ 0.91 and $P < 0.05$ considered meaningful, and then the CNC network was constructed using Cytoscape (v2.8.1). Co-expression relationships with significant *P*-values within a given upper percentile were entered as edges, while IL-6 and lncRNAs were entered as nodes in the CNC network. The co-expression relationship of each gene pair was estimated by the PCC.

Fluorescence *in situ* Hybridization (FISH) of lncSSBP1 in BEAS-2B Cells

Locked Nucleic Acid (LNA)-based probes were directed against the full length lncRNA sequence for lncSSBP1. The lncSSBP1 probe (sequence: 5′-TAAGAGTTGCTGCCAAGTATTTTCAAAATC-3′) was purchased from BioSense (Guangzhou, China). The FISH procedure was performed according to the BioSense instructions. Briefly, cell slides were fixed in 4% formaldehyde for 20 min and then washed twice for 5 min each with 0.1% diethy pyrocarbonate water. After digesting with proteinase K for 10 min, the slides were fixed in 1% formaldehyde for 10 min. The slides were sequentially subjected to dehydration using 70, 85, and 100% alcohol for 5 min each. Then, the slides were hybridized with the probes overnight (12–16 h) at 45°C in a humidified chamber. At the end of the hybridization period, the slides were sequentially treated with a warmed hybridization solution. Finally, the slides were counterstained with DAPI (Sigma, Shanghai, China) and scanned with Zeiss LSM 700 Meta confocal microscope (Oberkochen, Germany).

Construction of ceRNA Network

The interactions between lncSSBP1 and miRNA were predicted using DIANA-LncBase v2.0. Then the predicted targets were intersected with differentially expressed genes in the microarray data mentioned above. The lncSSBP1, miRNAs, and mRNAs were selected to construct the lncRNA-miRNA-mRNA regulatory network. The interactions and visualization were conducted by the Cytoscape software v3.4.0.

Overexpression and Knock Down of lncSSBP1

A lentiviral vector containing human lncSSBP1 was purchased from GenePharma (Suzhou, China) and used to overexpression lncSSBP1 (referred to as Lv-lncSSBP1). The negative control lentivirus (Lv-NC) was also purchased from GenePharma. An shRNA lentivirus vector containing the target sequence

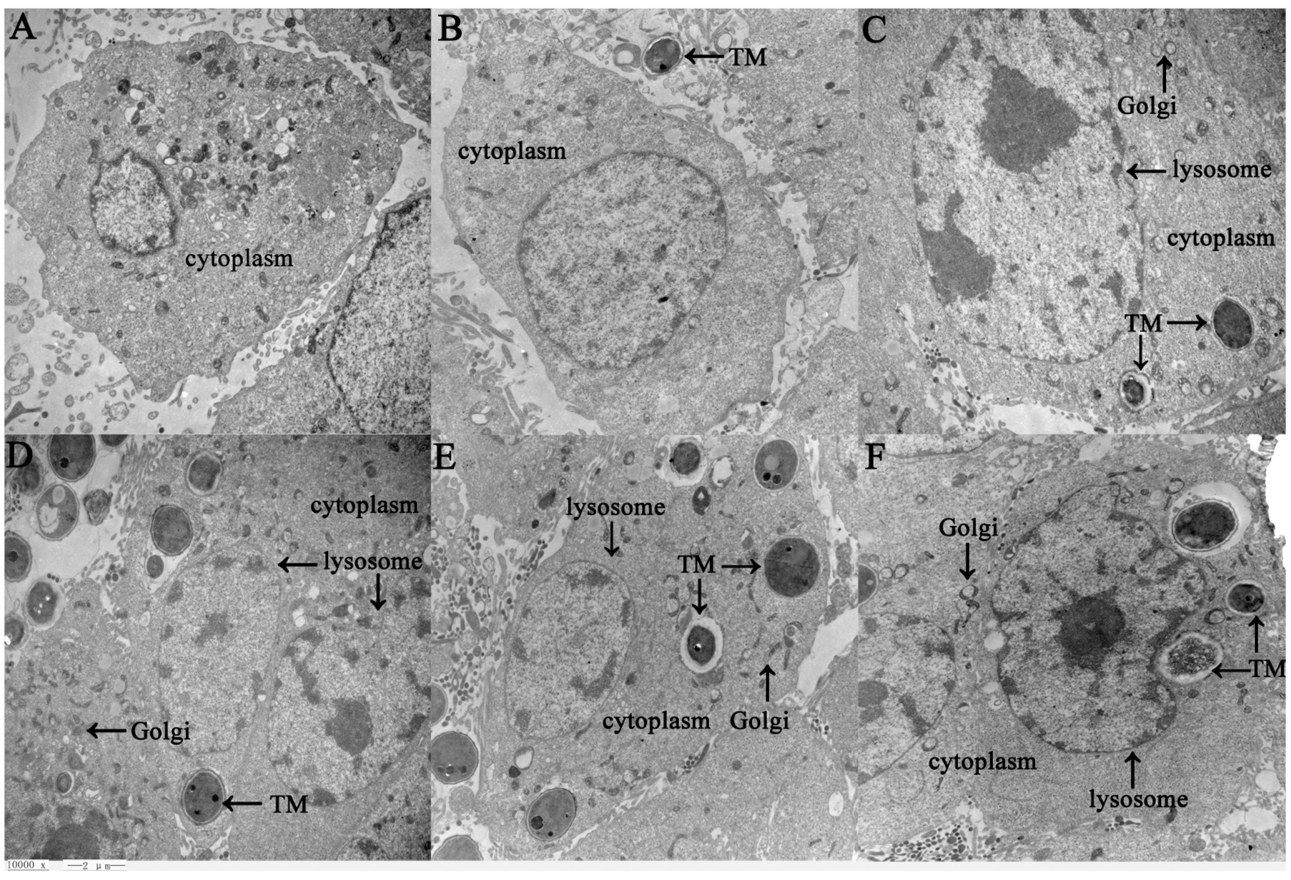

FIGURE 1 | Ultrastructure of BEAS-2B cells in response to TM infection as detected by transmission electron microscopy (10,000 x, Bar = 2 μm). BEAS-2B cells were incubated with TM spores for 1 h **(B)**, 2 h **(C)**, 3 h **(D)**, 4 h **(E)**, or 5 h **(F)**, and then changes between control cells **(A)** and treatment cells **(B–F)** were observed under transmission electron microscopy. Swollen organelles are marked with arrows.

of lncSSBP1 (5′-GGCGACAAGCAACAACAATCA-3′) was also used to knock down lncSSBP1 expression. The sequence was cloned into pGLVH1 (GenePharma, SuZhou, China) to generate Sh-lncSSBP1. A negative control lentivirus containing a non-targeting shRNA sequence (5′-TTCTCCGAACGTGTCACGT-3′; referred to as Sh-NC) was used as a control. All cloned sequences were verified by automated sequencing (GenePharma, SuZhou, China).

All lentiviral vectors, including Lv-lncSSBP1, Lv-NC, sh-lncSSBP1 and sh-NC, were transfected into 293FT cells for packaging. The virus particles were harvested 72 h after transfection of 293FT cells. For stable transfection, BEAS-2B cells were grown in six-well plates to 50% confluence, and 1 mL of viral supernatant was added with 1 μL polybrene. The interference efficiency of the template was verified by RT-PCR analysis.

RNA Pull-Down and Mass Spectrometry Analyses

LncSSBP1 template DNA was transcribed *in vitro* with T7 RNA polymerase (Roche, Basel, Switzerland) and purified with an RNeasy Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The purified 3′ end region of RNA was biotinylated using Pierce™ RNA 3′ End Desthiobiotinylation Kit (Thermo Fisher Scientific, Waltham, USA). Biotinylated RNA was incubated with streptavidin-agarose beads for 2 h at room temperature. Then the beads were washed briefly three times, and the retrieved protein was detected by spectrometry (Sagene Biotechnology, Guangzhou, China).

RNA Immunoprecipitation Assays

RNA immunoprecipitation was performed according to the instructions of the Magna RIP™RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cells were collected using a cell scraper and lysed using RIP lysis buffer containing proteinase inhibitors and ribonuclease inhibitors. Subsequently, whole cell lysates were mixed with magnetic beads conjugated with human anti-hnRNPK antibody (Abcam, Shanghai, China), or negative control IgG (Abcam, Shanghai, China) at 4°C overnight. After immunoprecipitation, the protein A/G-beads were washed with washing buffer. Finally, the immunoprecipitation products were collected. Immunoprecipitated RNA was purified and then subjected to qRT-PCR analysis to detect the relative levels of lncSSBP1 and IL-6. 7SK expression served as a positive internal control.

Statistical Analysis

Results are shown as the mean ± standard deviation (SD) of three independent experiments for each group. Statistical comparisons were conducted using SPSS20.0, and significance was assessed by the two-tailed Student's *t*-test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

TM Infection Induces Morphological Changes in BEAS-2B Bronchial Epithelial Cells

Human bronchial epithelial BEAS-2B cells were incubated with TM conidia at a ratio of 1:20 for 1–5 h, and then observed under a transmission electron microscope. After co-culture for 2 h, TM spores were phagocytized by BEAS-2B cells. With prolonged culture time, the number of phagocytic spores increased, and the phagocytic vacuoles became aggregated. Over time, the amount of intracellular cytoplasm and organelles increased significantly, and the Golgi, lysosomes and other organelles appeared swollen. Pseudopods extended out of the lysosomes, and the spores appeared damaged to a certain degree (**Figure 1**). These findings verify that TM induces observable pathological changes in a human bronchial epithelial cell culture model.

Expression of lncRNA NR_046269 and IL-6 Are Negatively Correlated in the Response to TM Infection

To identify regulatory pathways that may contribute to the pathogenic response to TM infection, we re-examined the lncRNA and mRNA expression profiles from our previous

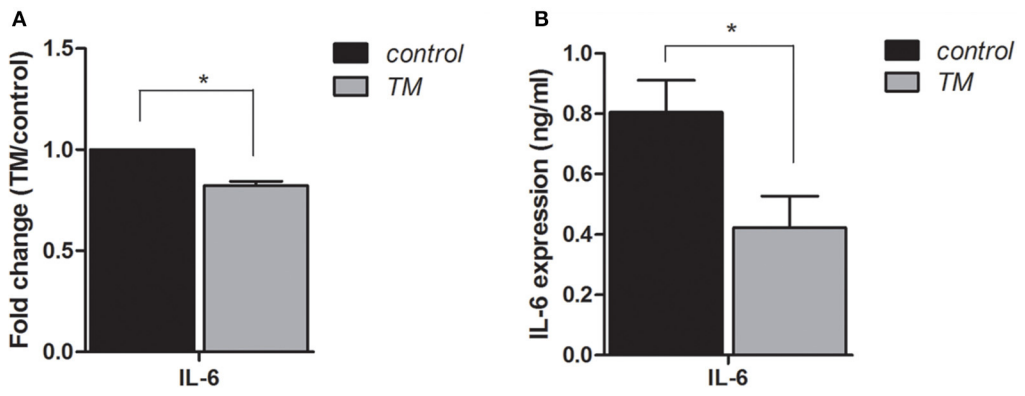

FIGURE 2 | Expression levels of IL-6 in BEAS-2B cells are down-regulated by infection with TM for 4 h. qRT-PCR **(A)** and ELISA **(B)** were performed to determine the expression levels of IL-6, *$P < 0.05$.

study (15), in which BEAS-2B cells were infected by TM spores for 4 h. We noted that among 519 lncRNAs and 329 mRNAs that were significantly differentially expressed (Fold change ≥ 1.5 or ≤−1.5, $P < 0.05$). Of these differentially expressed genes, we found that IL-6 expression was down-regulated. Given the key role of IL-6 in mediating inflammatory pathways and processes (12–14), we further examined its expression pattern by qRT-PCR and ELISA. The results verified that the expression of IL-6 is down-regulated in TM-infected cells, which is consistent with microarray data (**Figure 2**).

To examine potential mechanisms by which IL-6 is down-regulated by TM, we constructed a CNC network between IL-6 and the differentially expressed lncRNAs. The co-expression relationship of each gene pair was estimated by the PCC. In the CNC network, IL-6 was associated with 350 lncRNAs ($|PCC| \geq 0.91$, $P < 0.05$), of which 102 pairs presented as positive correlations and 248 pairs presented as negative correlations. Notably, IL-6 was highly negatively correlated with lncRNA NR_046269 (PCC = −0.998, **Figure 3**), indicating a potential role for this lncRNA in regulating IL-6 expression in response to TM. Since lncRNA

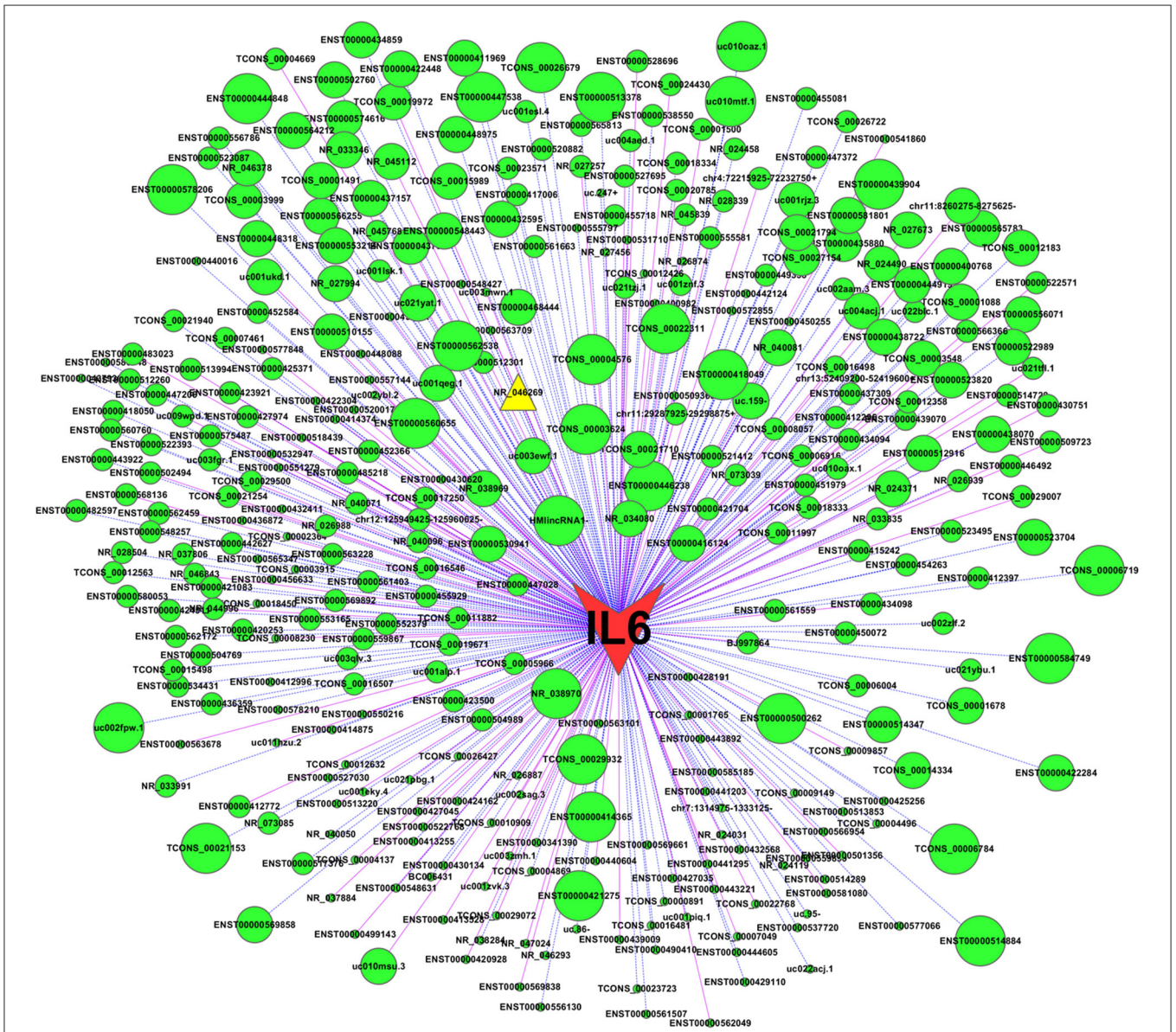

FIGURE 3 | CNC network between IL-6 and differentially expressed lncRNAs. Using expression data for IL-6 mRNA and lncRNAs in TM-infected BEAs-2B cells, a CNC network was constructed using Cytoscape (v2.8.1). |PCC| ≥ 0.91 and $P < 0.05$ was identified as significant. Nodes represent correlations between IL-6 and lncRNAs, while edges represent co-expression relationships. Solid pink lines represent positive co-relationships and blue dotted lines represent negative. Larger node sizes indicate more extensive relationships between IL-6 and the lncRNAs.

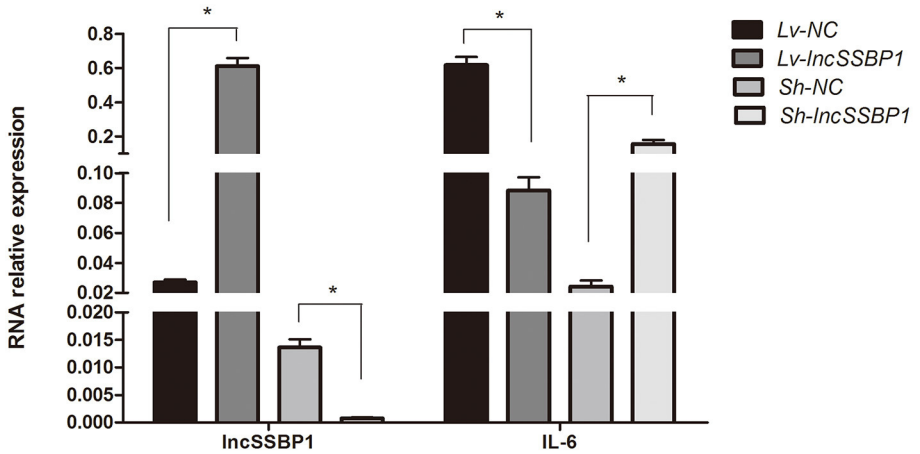

FIGURE 4 | Overexpression and knock down of lncSSBP1 in BEAS-2B cells inversely regulates IL-6 expression. **(Left)** qRT-PCR was performed to verify lncSSBP1 overexpression or knockdown in response to lncSSBP1 (Lv-lncSSBP1) or control (Lv-NC) expression lentivirus, and lncSSBP1 (Sh-lncSSBP1) or control (Sh-NC) shRNA lentivirus. **(Right)** At the same time, the relative expression levels of IL-6 were detected, $^*P < 0.05$.

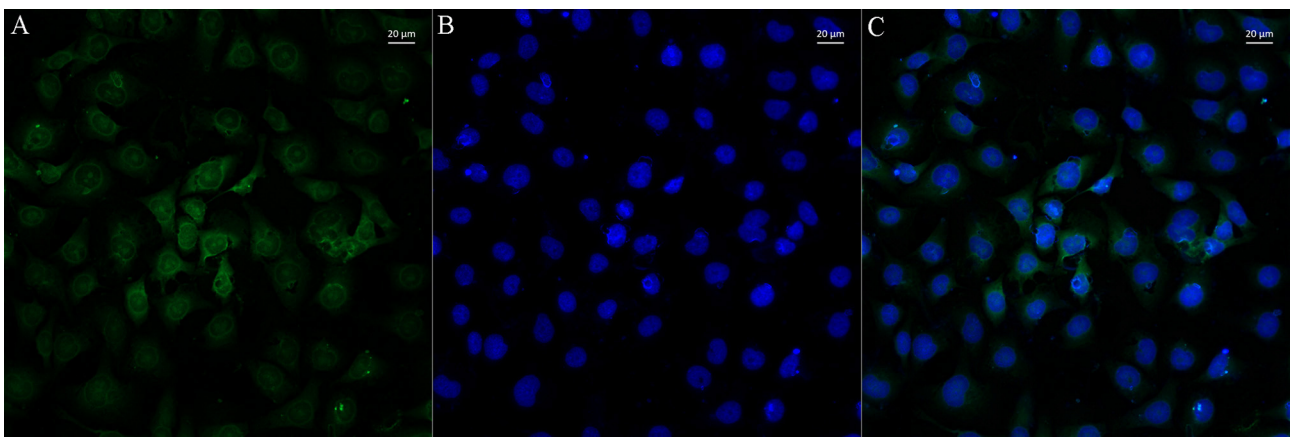

FIGURE 5 | LncSSBP1 is localized to the nucleus in BEAS-2B cells. **(A)** Green fluorescence of lncSSBP1 (excitation wavelength 550 nm and emission wavelength 570 nm) was detected by laser confocal microscopy. **(B)** The nuclei were DAPI counterstained (blue color; excitation wavelength 360 nm and emission wavelength 470 nm). **(C)** Overlay of **(B)** on **(A)**.

NR_046269 is adjacent to the coding gene single stranded DNA binding protein 1 (SSBP1), and partially overlaps with its transcribed region, we designated this lncRNA as lncSSBP1.

LncSSBP1 Negatively Regulates IL-6 Expression

To directly investigate whether lncSSBP1 regulates IL-6 expression, we used lentiviral vectors to overexpress and knock down lncSSBP1 in BEAS-2B cells. qRT-PCR results showed that the relative IL-6 mRNA expression level was significantly decreased after overexpression of lncSSBP1. Conversely, the IL-6 mRNA level was significantly increased after knock down of lncSSBP1 ($P < 0.05$, **Figure 4**). These results provide evidence that lncSSBP1 has a negative regulatory effect on IL-6.

LncSSBP1 Localizes to the Nucleus in BEAS-2B Cells

To further explore the function of lncSSBP1, FISH was performed. The results show that lncSSBP1 was mainly distributed in the nucleus with a small amount distributed in the cytoplasm around the nucleus (**Figure 5**). These findings are consistent with a nuclear regulatory role for lncSSBP1.

LncSSBP1 Is Unlikely to Regulate IL-6 by Functioning as a Competing Endogenous RNA (ceRNA)

Using computational approaches, we calculated the likelihood of lncRNA-microRNA-mRNA interaction. A total of 129 microRNAs were retrieved as candidate interacting partners of lncSSBP1 (**Figure 6**). However,

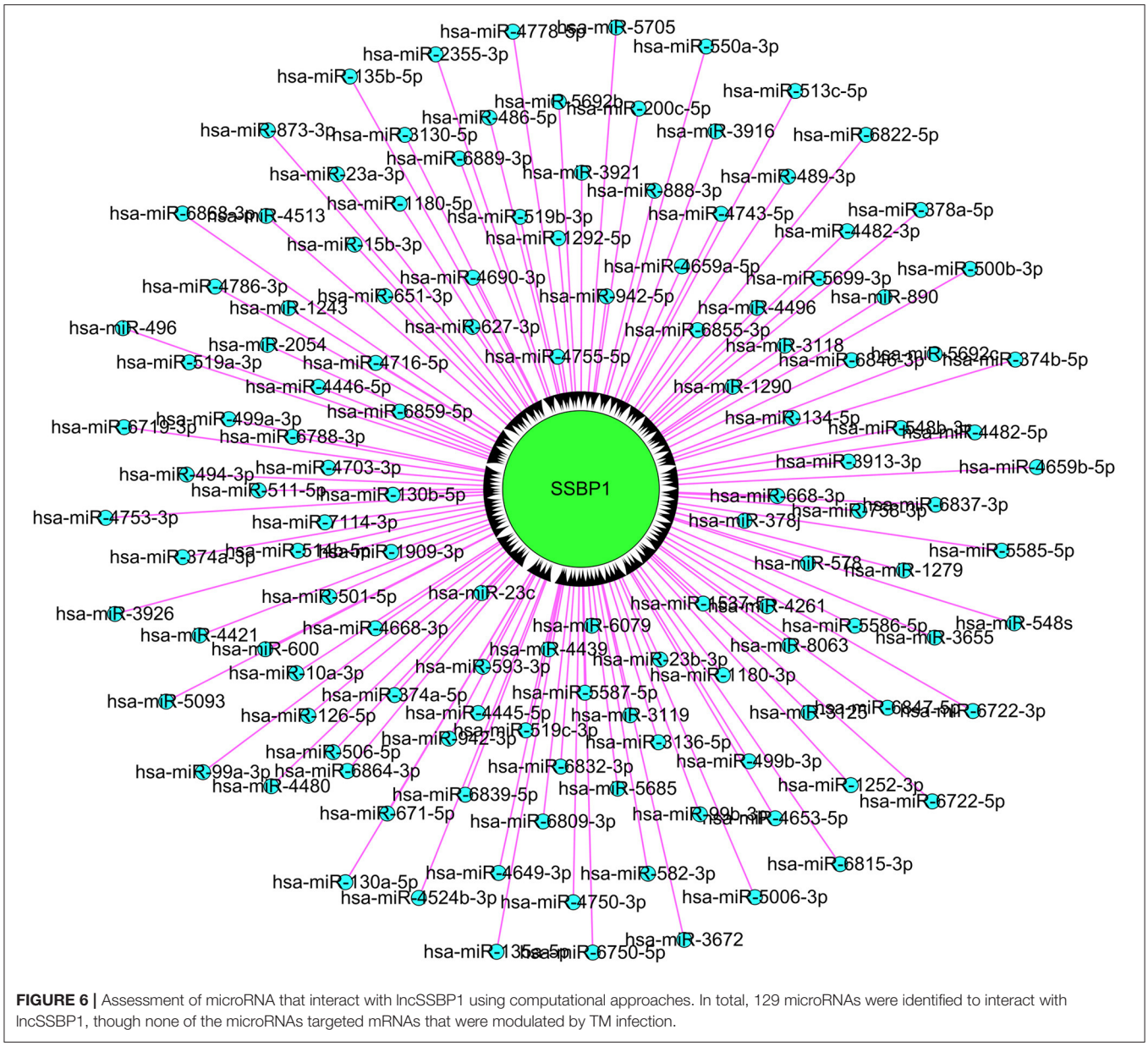

FIGURE 6 | Assessment of microRNA that interact with lncSSBP1 using computational approaches. In total, 129 microRNAs were identified to interact with lncSSBP1, though none of the microRNAs targeted mRNAs that were modulated by TM infection.

none of these microRNAs were predicted to interact with differentially expressed mRNAs, including IL-6 in our profiled data. These results suggest that lncSSBP1 is unlikely to regulate IL-6 expression by functioning as a ceRNA.

LncSSBP1 RNA Inhibits IL-6 Expression via Interaction With hnRNPK

To identify alternate methods by which lncSSBP1 may regulate IL-6 expression, we performed RNA pull down assays followed by mass spectrometry of proteins that bind directly or indirectly to lncSSBP1. In total, 284 proteins were obtained (**Table S2**), however, this set of lncSSBP1-interacting partners did not include IL-6 transcription factors. Therefore, we sought to identify other interactions that may affect IL-6 expression. Using computational approaches, we determined that hnRNPK is among the higher-scoring proteins. As hnRNPK belongs to a class of nuclear RNA-binding proteins that interact with precursor mRNAs (18–21), we reasoned that it could potentially mediate the function of lncSSBP1.

To examine the specificity of the interaction between lncSSBP1 and hnRNPK in BEAS-2B cells, we performed RIP assays with anti-hnRNPK antibodies or control IgG, followed by qRT-PCR. The relative enrichment of lncSSBP1 in the IP-hnRNPK group was remarkably higher than in IgG group. Furthermore, the relative expression of IL-6 mRNA in the IP-hnRNPK group was also significantly higher than in the IgG group (**Figure 7**). These results indicate that hnRNPK interacts with both lncSSBP1 and IL-6, which provides a pathway by which lncSSBP1 may regulate IL-6.

DISCUSSION

In the process of infection, pathogens tend to induce the body to produce immunosuppressive factors to evade immune attacks, so as to promote their own survival. Immune evasion has been demonstrated in TM pathogenesis *in vivo* (22, 23), though its exact mechanisms are poorly understood. Like *M. tuberculosis*, TM is engulfed by monocytes/macrophages and lurks in the reticuloendothelial system (23). Furthermore, TM can survive oxidative stress, heat shock, nutrient starvation, pH, enzymes, and dimorphic morphologic shifts (22, 24, 25). Studies have indicated that IL-6 and its signaling pathways are involved in immune evasion (26–28). For most early stages of infections, the expression of IL-6 is elevated. However, for some infections, the expression of IL-6 is reduced. For example, *M. tuberculosis* secretes lower levels of IL-6 when infected in adipocytes, which may help *M. tuberculosis* to lurk in fat cells (29). Furthermore, for *Chlamydia trachomatis*, there are no differences in the expression levels of IL-6, TNF-α, and CXCL8 for early stage infection of cervical epithelial cells, indicating that it can also escape from the strong pro-inflammatory responses, thus facilitating its adaptation to the intracellular microenvironment (30). In our previous and present studies (15), using microarray, qRT-PCR and ELISA, we identified that IL-6 were all significantly decreased in human bronchial epithelial cells incubated with TM conidia for 4 h, while using transmission electron microscope, we observed that the number of conidia and organelles was significantly increased in the same cell model, and the Golgi, lysosomes and other organelles appeared swollen over time. So we supposed that TM induced cytoplasmic organelles injury and reduction of IL-6 may facilitate its invasion.

LncRNAs are major participants in gene expression regulatory networks, and their precise sequence and natural domains define their molecular mode of action and specific execution of their biological functions (31). They regulate transcription, splicing, nucleic acid degradation and translation through RNA-RNA, RNA-DNA, or RNA-protein interactions (32). LncRNAs have been disclosed to have critical roles in modulating immune responses, but the majority of them remain uncharacterized (33). Using microarray and CNC analysis, we found that lncSSBP1 is highly negatively correlated with IL-6 expression after TM infection, suggesting that lncSSBP1 may have a negative regulatory effect on IL-6 expression. Subsequently, cell transfection experiments demonstrated that IL-6 expression was down-regulated when lncSSBP1 was overexpressed, while IL-6 was up-regulated when lncSSBP1 was knocked down.

To explore the potential mode of action of lncSSBP1, we examined its subcellular localization. Using FISH, we determined that most of the lncSSBP1 was localized to the nucleus, with a small amount in the cytoplasm, indicating that lncSSBP1 may carry out its activities through the interaction with microRNAs (as a ceRNA), transcription factors, heterogeneous ribonucleoproteins (hnRNPs), or chromatin-modifying complexes in the nucleus.

We used computational approaches to assess the likelihood of lncRNA-microRNA-mRNA interactions. However, we did not predict any microRNA interactions with IL-6 mRNA, suggesting that lncSSBP1 is not likely to function as a ceRNA in regulating IL-6 expression.

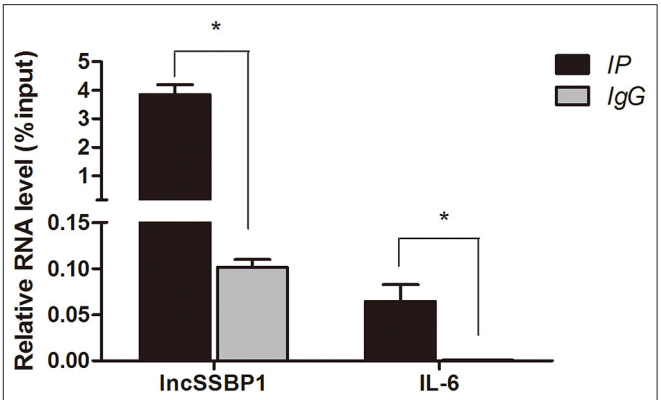

FIGURE 7 | hnRNPK interacts with lncSSBP1 and IL-6 mRNA in an RNA immunoprecipitation assay. Anti-hnRNPK was used to immunoprecipitate hnRNPK complexes, which were evaluated for lncSSBP1 and IL-6 mRNA content by qRT-PCR. RNA levels are presented as relative expression compared to the input group, $^*P < 0.05$.

To further evaluate potential mechanisms of lncSSBP1, we performed RNA pull down and mass spectrometry experiments of lncSSBP1 interacting proteins. We did not find IL-6 transcription factors among the proteins pulled down by lncSSBP1, and we therefore looked for other proteins that might affect IL-6 expression. Among the detected proteins, hnRNPK was identified as a candidate target that may be involved in IL-6 mRNA processing.

hnRNPK is a component of heterogeneous nuclear ribonucleoprotein complexes (hnRNPs) that interacts with precursor mRNAs and are by far the most important molecular chaperones that mediate the functions of lncRNAs (18–21). As a component of hnRNPs, hnRNPK is reported to participate in transcription, splicing, editing, localization, and degradation of mRNAs and maturation of microRNA precursors (34, 35). Additionally, there is evidence that hnRNPK contributes to the regulation of RNA 3′-end processing. In THP-1 monocytes, hnRNPK is shown to control cytoplasmic COX-2 mRNA stability by modulating its binding to the COX-2 promoter and COX-2 3′-UTR (36). RIP-Chip analyses demonstrated 1,901 mRNAs that were differentially bound to hnRNPK, which interacts specifically with a sequence in the transforming growth factor-β-activated kinase 1 (TAK1) mRNA 3′-UTR in LPS-activated macrophages. Reduction of hnRNPK increases endogenous TAK1 mRNA translation, resulting in enhancement of TNF-α, IL-1β, and IL-10 mRNA expression (37). In this report, hnRNPK was found to significantly enrich lncSSBP1 in pull down assays, indicating that lncSSBP1 binds specifically to hnRNPK. HnRNPK also bound to IL-6 mRNA, suggesting that lncSSBP1 may repress basal expression levels of IL-6 mRNA through its interaction with hnRNPK. These findings provide a pathway by which lncSSBP1 may regulate the expression of IL-6 during TM infection to enhance immune evasion.

In summary, IL-6 expression is down-regulated during TM infection in bronchial epithelial cells. LncSSBP1 has an overall

negative effect on IL-6 expression, which may be beneficial to TM immune evasion. Our findings suggest that lncSSBP1 may perform its regulatory activity on IL-6 mRNA by specifically interacting with hnRNPK. Additional mechanisms by which lncSSBP1 affects IL-6 remain to be addressed in future studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZH and XL designed the research, interpreted the data, and gave final approval of the version to be published. YiL contributed to computer programs, data analysis, and drafted the manuscript. YiL, HC, SL, and YuL performed the majority of the experiments and of the data collection. GL, JB, and HL provided suggestions during manuscript preparation and revised the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02977/full#supplementary-material

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Using Interleukin 6 and 8 in Blood and Bronchoalveolar Lavage Fluid to Predict Survival in Hematological Malignancy Patients With Suspected Pulmonary Mold Infection

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Background: Molds and other pathogens induce elevated levels of several cytokines, including interleukin (IL)-6 and IL-8. The objective of this study was to investigate the prognostic value of IL-6 and IL-8 as well as fungal biomarkers in blood and bronchoalveolar lavage fluid (BAL) for overall survival in patients with underlying hematological malignancies and suspected mold infection.

Methods: This cohort study included 106 prospectively enrolled adult cases undergoing bronchoscopy. Blood samples were collected within 24 h of BAL sampling and, in a subset of 62 patients, serial blood samples were collected up until 4 days after bronchoscopy. IL-6, IL-8, and other cytokines as well as galactomannan (GM) and β-D-glucan (BDG) were assayed in blood and BAL fluid and associations with overall mortality were assessed at the end of the study using receiver operating characteristic (ROC) curve analysis.

Results: Both blood IL-8 (AUC 0.731) and blood IL-6 (AUC 0.699) as well as BAL IL-6 (AUC 0.763) and BAL IL-8 (AUC 0.700) levels at the time of bronchoscopy were predictors of 30-day all-cause mortality. Increasing blood IL-6 levels between bronchoscopy and day four after bronchoscopy were significantly associated with higher 90-day mortality, with similar findings for increasing IL-8 levels. In ROC analysis the difference of blood IL-8 levels between 4 days after bronchoscopy and the day of bronchoscopy had an AUC of 0.829 (95%CI 0.71–0.95; $p < 0.001$) for predicting 90-day mortality.

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Conclusions: Elevated levels of IL-6 and IL-8 in blood or BAL fluid at the time of bronchoscopy, and rising levels in blood 4 days following bronchoscopy were predictive of mortality in these patients with underlying hematological malignancy who underwent bronchoscopy for suspected mold infection.

Keywords: hematologic malignancy, invasive mold infection, interleukin-6, interleukin-8, prognosis

INTRODUCTION

Patients with hematologic malignancies are immunocompromised with increased rates of hospital admissions and, in particular, admissions to the intensive care unit (ICU) (1). Factors that increase the risk of admission to the ICU include pneumonia caused by invasive mold infections (IMI) or other pathogens (2). IMI, including invasive aspergillosis (IA), are associated with high morbidity and mortality among patients with underlying hematological malignancies (3–7). Prognosticating survival in patients at risk for IMI remains difficult (8), although prompt identification of those most at risk for severe complications and death and early initiation of antifungal or other anti-infective therapy could lead to better outcomes (9).

Aspergillus spp. have been shown to induce T-helper cell subsets resulting in elevated levels of several cytokines (10, 11) and recent studies have indicated—after adjusting for multiple covariates also associated with higher cytokine levels—that particularly Interleukin (IL)-8 and IL-6 may show promise as diagnostic markers (12, 13). Our own work suggests that elevated levels of IL-8 in patients presenting with suspected pulmonary infection have excellent specificity (>90%) for detecting IMI (14), however whether these cytokines may also predict overall mortality and whether serial measurement of this cytokines may increase their prognostic potential remains unknown.

The objective of this analysis was to determine the potential of variations in IL-6 and IL-8, as well as established fungal biomarkers, to predict overall mortality in patients with underlying hematological malignancies and suspicion of pulmonary mold infection in a setting that uses mold-active prophylaxis.

MATERIALS AND METHODS

This prospective cohort study comprised paired routine serum and BAL samples obtained on the same day from cases with underlying hematological malignancies who underwent routine bronchoscopy due to suspicion of pulmonary mold infections. The decision was based on suspicious or non-specific radiological findings in chest computed tomography, with or without clinical laboratory findings including fungal biomarker levels. Investigators had no influence on clinical interventions (e.g., bronchoscopy) and treatment of the enrolled patients. The diagnostic potential of several biomarkers and cytokines measured in same day BAL and blood samples obtained as part of this cohort study for diagnosing IMI has been previously published (13, 14). However, the present analysis focuses on the overall prognostic potential of several cytokines and biomarkers in clinical outcomes of IMI. Approximately halfway through the study, the protocol was modified to include permission to use routinely-collected surplus plasma samples stored in the hospital laboratory for 4 days following collection. These longitudinal samples have not previously been published.

IA and IMI were graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG) (15).

Study Cohort

Participants undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Graz, Austria, between April 2014 and July 2017. Key inclusion criteria were (i) adult patients with (ii) underlying hematological malignancy who were (iii) at risk for IMI according to the attending clinicians discretion (e.g., febrile neutropenia, induction chemotherapy for acute myeloid leukemia, allogeneic stem cell transplantation), had (iv) a BAL sample obtained due to clinical suspicion of pulmonary infection, and (v) an order of fungal biomarkers from BAL [i.e., galactomannan (GM) and in a subset also panfungal or Mucorales specific polymerase chain reaction]. All patients who met inclusion criteria between April 2014 and July 2017 and signed an informed consent were included in the cohort. After informed consent was obtained, serum and whole blood samples were collected within 24 h of bronchoscopy. In the last 62 patients enrolled in the study, serial daily plasma samples were also obtained from blood samples drawn as part of routine clinical care from 4 days before bronchoscopy until 4 days after bronchoscopy. Because Investigators had no influence on blood sample drawn besides the same-day samples, sample size varied between 2 and 9 plasma samples per case. These were stored at 4°C for up to 4 days before processing for storage at −70°C and analysis.

Biomarker Testing

Conventional culture as well as BAL and serum GM concentrations (Platelia enzyme immunoassay; Bio-Rad Laboratories, Vienna, Austria) were prospectively determined in clinical routine at the Medical University of Graz. Given that the vast majority of patients received mold-active antifungals at the time of bronchoscopy, cut-offs of 0.5 GM optical density index (ODI) where used for serum and BAL, following previous

evidence that the 0.5 ODI cutoff is preferable in patients on mold-active antifungals (16).

β-D-glucan (BDG) testing was performed in part prospectively and in part retrospectively at the Medical University of Graz, using the commercially available Fungitell® assay (Cape Cod Diagnostics, Falmouth, MA, USA) with an adopted protocol suitable for use on a routine BCS XP® coagulation analyzer, as described previously (17). BDG testing was only performed in serum samples, as BAL BDG testing yields very low specificity due to non-pathogenic *Candida* colonization in the lungs and high BDG values (18–20). For serum BDG we used the recommended cut-off of ≥ 80 pg/mL to define positivity.

All blood (i.e., serum and plasma) and BAL isolates used in this study were frozen to −70°C after processing and stored for batched analysis. IL-6 and IL-8 concentrations were determined in serum, plasma, and BAL samples at the Center for Medical Research of the Medical University of Graz, Austria, between 09/2016 and 10/2017 with a personalized ProcartaPlex® immunoassay (eBioscience, Vienna, Austria) as previously described (13).

Investigators measuring biomarkers and cytokine levels were blinded toward clinical and demographic information of the patients.

Assessing Mortality

All patients enrolled in the study were followed clinically on initial admission and their medical records were reviewed after discharge. Autopsies were not routinely performed or requested as part of the study and therefore the absolute rate of autopsies on patients in the study was very low—likely reflecting the low cultural predilection for autopsy in the study country.

Our study was conducted in accordance with the Declaration of Helsinki, 2013, Good Clinical Practice. The study protocol was approved by the local ethics committee, Medical University Graz, Austria (EC-numbers 25-221 and 23-343), and registered at ClinicalTrials.gov (Identifier: NCT02058316 and NCT01576653). Informed consent was obtained from all study participants. Statistical analysis was performed using SPSS, version 25 (SPSS Inc., Chicago, IL, USA). For continuous data, including cytokine levels, receiver operating characteristic (ROC) curves analyses were performed and area under the curve (AUC) values are presented including 95% confidence intervals (95% CI) for the 30-, 90-, and 180-day overall mortality outcomes (*p*-values were not corrected for multiple comparisons). Optimal cut-offs for cytokines discriminating in patients who died within 30 days vs. those who survived were calculated using the Youden's index. Two-sided p-value < 0.05 was taken as cut-off for statistical significance.

RESULTS

Study Cohort

In total, 122 participants undergoing bronchoscopy were prospectively enrolled between April 2014 and July 2017. A total of 16 cases had to be excluded due to the following reasons: (i) same day blood samples (i.e., collected within 24-h) were not available ($n = 13$); (ii) BAL volume after routine testing was insufficient for further diagnostic work up within the study protocol ($n = 2$); (iii) hematological malignancy was suspected but not confirmed because of mortality within days of admission ($n = 1$). After exclusion of these 16 cases, 106 patients remained in the final analysis. Patients' characteristics are displayed in **Table 1**. Mortality was 16% (17/106) at 30 days, 27.4% (29/106) at 90 days, and 42.5% (45/106) at 180 days after study enrollment and bronchoscopy. In those with mold infections, 30-day mortality was 36% for probable/proven IA, 16% for possible IA and 13% for those without evidence for IA (for all probable/possible/no IMI 33, 12, and 13%, respectively).

Overall patients who died within 30 days were more frequently neutropenic at the time of bronchoscopy, had more frequently received T-cell suppressants, had more frequently probable or proven IMI and had received more frequently mold active prophylaxis or treatment at the time of bronchoscopy (**Table 1**). Of the 17 cases who died within 30 days of bronchoscopy (median 14 days after bronchoscopy, range 1–30 days), autopsy was performed in four cases, revealing progression of acute myeloid leukemia as cause of death in two cases, while cause of death was infectious (organized viral pneumonia, multi organ failure) in the other two cases. Of the other 13 cases who did not undergo autopsy, five had probable IMI, two possible IMI, two viral pneumonias, and each one bacterial pneumonia or systemic bacterial infection. Neutropenia and receipt of mold-active antifungals were also more frequent in those who died within 90 days, in fact every single patient who died within 90 days had received a mold-active antifungal at the time of bronchoscopy while this was 74% of patients who survived to day 90 ($p = 0.002$).

Prognostic Potential of Blood and BAL Cytokines at the Time of Bronchoscopy

In ROC curve analysis, serum IL-8 was a significant predictor of 30-day overall mortality, followed by serum IL-6, while serum GM and serum BDG were not significant predictors (**Table 2**). In BAL IL-6 and IL-8 were significant predictors of overall 30-day mortality, while GM was not (**Table 2**). Both serum IL-8 and serum IL-6 as well as BAL IL-6 were also significant predictors of 90- and 180-day cumulative overall mortality, although AUCs were lower when compared to 30-day mortality, and significance driven mostly by the predictive potential for 30-day mortality (**Table 2**). When focusing only on participants who died within 30- and 90-days, or between 90- and 180-days, AUCs were highest for serum IL-8 (AUC 0.578 and 0.592, respectively), followed by serum IL-6 (AUC 0.564 for both; all $p > 0.2$). Boxplots of serum and BAL IL-8 and IL-6 levels in those who died and those who survived are displayed in **Figure 1**. For prediction of 30-day mortality, optimal cut-offs were serum IL-8 > 13.93 pg/mL (82.4% sensitivity, 61.8% specificity), serum IL-6 > 165 pg/mL (52.9% sensitivity, 87.6% specificity), BAL IL-8 > 1,111 pg/mL (64.7% sensitivity, 69.7% specificity), and BAL IL-6 > 43.95 pg/mL (100% sensitivity, 46.1% specificity).

Sub-analyses for 30-day mortality found that (i) among those with possible, probable or proven IMI serum IL-8 (AUC

TABLE 1 | Demographic data, underlying diseases, and infections in cases who died within 30 and 90 days after bronchoscopy vs. those who survived.

Demographic data, underlying diseases and other characteristics at the time of sampling ($n = 106$)		Mortality at day 30 ($n = 17$)	Survival at day 30 ($n = 89$)	*p*-value*	Mortality at day 90 ($n = 29$)	Survival at day 90 ($n = 77$)	*p*-value*
Sex	Female	6 (35%)	47 (53%)	>0.2	10 (34%)	43 (56%)	0.050
	Male	11 (65%)	42 (47%)		19 (66%)	34 (44%)	
Age [years]	Median (range)	55 (33–66)	59 (26–85)	0.13	56 (27–78)	58 (26–85)	>0.2
Underlying diseases	AML/MDS	8 (47%)	43 (48%)	>0.2	18 (62%)	33 (43%)	>0.2
	NHL	2 (12%)	22 (25%)		4 (14%)	20 (26%)	
	MM	2 (12%)	7 (8%)		2 (7%)	7 (9%)	
	ALL	2 (12%)	9 (10%)		2 (7%)	9 (12%)	
	Others[#]	3 (18%)	8 (9%)		3 (10%)	8 (10%)	
Other conditions	Allogeneic stem cell transplantation	5 (29%)	23 (26%)	>0.2	8 (28%)	20 (26%)	>0.2
	Autologous stem cell transplantation	1 (6%)	9 (10%)	>0.2	1 (3%)	9 (12%)	>0.2
	Graft vs. host disease	3 (18%)	13 (15%)	>0.2	5 (17%)	11 (14%)	>0.2
	Neutropenia (<500 μL) on day of BAL	12 (71%)	34 (38%)	**0.017**	18 (62%)	28 (36%)	**0.017**
	T-Cell Suppressants within 3 months of BAL	5 (29%)	9 (10%)	**0.047**	7 (24%)	7 (9%)	0.055
	Systemic corticosteroid treatment within 14 days of sampling	5 (29%)	25 (28%)	>0.2	8 (28%)	22 (29%)	>0.2
Invasive fungal infections	Probable/proven IMI	6 (35%)	12 (13%)	**0.039**	6 (21%)	12 (16%)	>0.2
	Possible IMI	3 (18%)	22 (25%)	>0.2	9 (31%)	16 (21%)	>0.2
	Probable/proven invasive aspergillosis	4 (24%)	7 (8%)	0.07	4 (14%)	7 (9%)	>0.2
	Antimould prophylaxis[$]/treatment	17 (100%)	69 (78%)	**0.030**	29 (100%)	57 (74%)	**0.002**
Other infections	Positive diagnostic test for relevant bacterial pathogens, pneumocystis or toxoplasma in BAL	3 (18%)	18 (20%)	>0.2	5 (17%)	16 (21%)	>0.2
	Positive diagnostic test for bacterial infections in other samples (blood culture/biopsies/urine) within 14 days of sampling	5 (29%)	19 (21%)	>0.2	9 (31%)	15 (19%)	>0.2
	Positive diagnostic test for viral infections within 14 days of sampling	6 (35%)	32 (36%)	>0.2	10 (34%)	28 (36%)	>0.2

**Bold indicates p-values that met statistical significance.*

#Included 5 chronic lymphoid leukemia (CLL); 3 primary myelofibrosis; 2 Hodgkin's lymphoma, and 1 anaplastic anemia.

$Breakdown of antifungal prophylaxis: 19 received voriconazole, 18 received posaconazole prophyaxis at the time of sampling.

ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; BAL, bronchoalveolar lavage fluid; GvHD, Graft vs. host disease; IA, invasive aspergillosis; IMI, invasive mold infection; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-hodgkin lymphoma; SCT, stem cell transplantation.

0.650), BAL IL8 (AUC 0.693), and BAL IL6 (AUC 0.631) had prognostic potential, while serum-IL-6 did not; (ii) among those with bacterial infections, serum IL-8 (AUC 0.685), BAL IL-8 (AUC 0.756), and BAL IL-6 (AUC 0.685) had prognostic potential, while serum IL-6 did not; (iii) among those with viral infections, only BAL levels of IL-8 (AUC 0.734) and IL-6 (AUC 0.677) had prognostic potential, while serum levels did not; (iv) among those with neutropenia at the time of bronchoscopy, serum levels of IL-6 (AUC 0.691) and IL-8 (AUC 0.642) had some prognostic potential while BAL levels had not; in contrast, BAL IL-8 (AUC 0.920) and BAL IL-6 (AUC 0.862) had very strong prognostic potential among non-neutropenic patients; (v) among those on corticosteroids, BAL IL-6 (AUC 0.831) and BAL IL-8 (AUC 0.728) levels had stronger prognostic potential than serum levels; and (vi) among female patients serum IL-8 (AUC 0.862), serum IL-6 (AUC 0.771), BAL IL-8 (AUC 0.812), and BAL IL-6 (AUC 0.801) had all strong prognostic potential.

TABLE 2 | Performance of cytokine levels*, Galactomannan (GM) and Beta-D-glucan (BDG) in serum and bronchoalveolar fluid (BAL) for differentiating cases who died within 30, 90, and 180 days of bronchoscopy vs. those who survived.

Sample	Biomarker or cytokine	Test performance for predicting overall 30 days mortality (17/106)				Test performance for predicting overall 90 days mortality (29/106)				Test performance for predicting overall 180 days mortality (45/106)			
		AUC	95% CI		*p*-value	AUC	95% CI		p-value	AUC	95% CI		*p*-value
			Lower bound	Upper bound			Lower bound	Upper bound			Lower bound	Upper bound	
BAL	BAL GM	0.484	0.318	0.649	0.833	0.477	0.355	0.600	0.720	0.509	0.397	0.620	0.882
	IL-6	***0.763***	***0.663***	***0.863***	***0.001***	***0.687***	***0.570***	***0.805***	***0.003***	***0.620***	***0.509***	***0.731***	***0.036***
	IL-8	***0.700***	***0.574***	***0.826***	***0.009***	0.603	0.483	0.724	0.102	0.536	0.424	0.647	0.534
Serum	Serum GM	0.646	0.477	0.814	0.058	0.571	0.443	0.700	0.259	0.538	0.424	0.653	0.503
	Serum BDG	0.563	0.403	0.724	0.411	0.406	0.279	0.533	0.139	0.446	0.333	0.559	0.346
	IL-6	***0.699***	***0.553***	***0.845***	***0.010***	***0.647***	***0.528***	***0.766***	***0.020***	***0.627***	***0.518***	***0.735***	***0.027***
	IL-8	***0.731***	***0.621***	***0.840***	***0.003***	***0.658***	***0.548***	***0.769***	***0.013***	***0.646***	***0.541***	***0.751***	***0.011***
	IL-10	0.557	0.408	0.706	0.459	0.502	0.380	0.625	0.971	0.514	0.402	0.626	0.808

**Only cytokines that have shown significant associations with mortality in the previously conducted nested case-control analysis matched for multiple covariates, including neutrophil status, immunosuppressant and concomitant viral and bacterial infections (13), were included in the primary analyses of this study.*

Significant differences ($p < 0.05$) are bold and italicized (p-values not corrected for multiple comparisons).

AUC, area under the curve; BAL, bronchoalveolar lavage fluid; CI, confidence interval; GM, galactomannan; BDG, beta-D-glucan; IA, invasive aspergillosis; IMI, invasive mold infection; IFN, interferon; IL, interleukin.

Kinetics of Blood IL-6 and IL-8 Before and After Bronchoscopy

In a subset of 62 participants, serial blood samples were obtained from 4 days preceding to 4 days following bronchoscopy (this particular subset had 30-day mortality of 14%, 90-day mortality of 28%, and 180-day mortality of 50%). Kinetics of serum IL-8 and IL-6 levels stratified by categories of overall mortality are displayed in **Figures 2**, **3** (23 samples tested for Day −4, 42 samples for Day −3, 46 samples for Day −2; 53 samples for Day −1; 62 samples for Day 0; 48 samples for Day +1; 52 samples for Day +2; 46 samples for Day +3; 49 samples for Day +4).

Between the day of bronchoscopy and 4 days following bronchoscopy (samples available from 49 participants on both time points), blood IL-6 levels increased in 14/49 (29%) participants, while blood IL-8 levels increased in 19/49 (39%) of participants. Increasing IL-6 levels at day four were significantly associated with higher 90-day mortality rates [7/13 (54%) died vs. 7/36 (19%) with stable or decreasing levels; $p = 0.031$], with similar findings for increasing IL-8 levels 4 days following bronchoscopy [9/18 (50%) died vs. 5/31 (16%); $p = 0.020$]. In ROC analysis, the difference of IL-8 levels between 4 days following bronchoscopy and the day of bronchoscopy had an AUC of 0.829 (95%CI 0.71–0.95; $p < 0.001$) for predicting 90-day mortality (AUC for IL-6 0.686; 95%CI 0.499–0.872; $p = 0.044$). AUC for IL-8 difference to predict 30-day mortality was 0.771 (95%CI 0.578–0.963; $p = 0.023$) and for 180-day mortality 0.680 (95%CI 0.529–0.831; $p = 0.031$); while differences in IL-6 levels were not significant predictors for 30- and 180-day mortality.

DISCUSSION

We evaluated prognostic potential of IL-6, IL-8, and several fungal biomarkers for overall mortality in a high-risk cohort of hematological malignancy patients (>80% of cases had received mold-active antifungals at the time of sampling) undergoing bronchoscopy for suspected pulmonary mold infection. Two major findings are evident. First, elevated levels of IL-6 or IL-8 in blood or BAL fluid at the time of bronchoscopy was associated with increased 30-day overall mortality. Second, increasing blood levels of IL-8 within the 4 days following bronchoscopy were highly predictive of overall 30- and 90-day mortality.

From prior studies, it is understood that cytokines are centrally involved in protective immunity against *Aspergillus* spp. and other molds (13, 21) and may therefore be used as an early biomarker for risk stratification regarding IA associated mortality (22). In the early stages of invasive aspergillosis (IA), conidia are killed by local alveolar macrophage and IL-8, also known as neutrophil chemotactic factor, is produced by these macrophages as well as neighboring epithelial cells as an important chemoattractant for neutrophils (11, 21). The mechanism of IL-8 increase during IA has also been studied *in vitro* where an up-regulation of gene transcription by *Aspergillus fumigatus* proteases was shown to cause increased release of IL-8 (as well as IL-6, which plays an important role in T cell recruitment) by A549 pulmonary epithelial cells and primary epithelial cells (16). Other studies have shown that

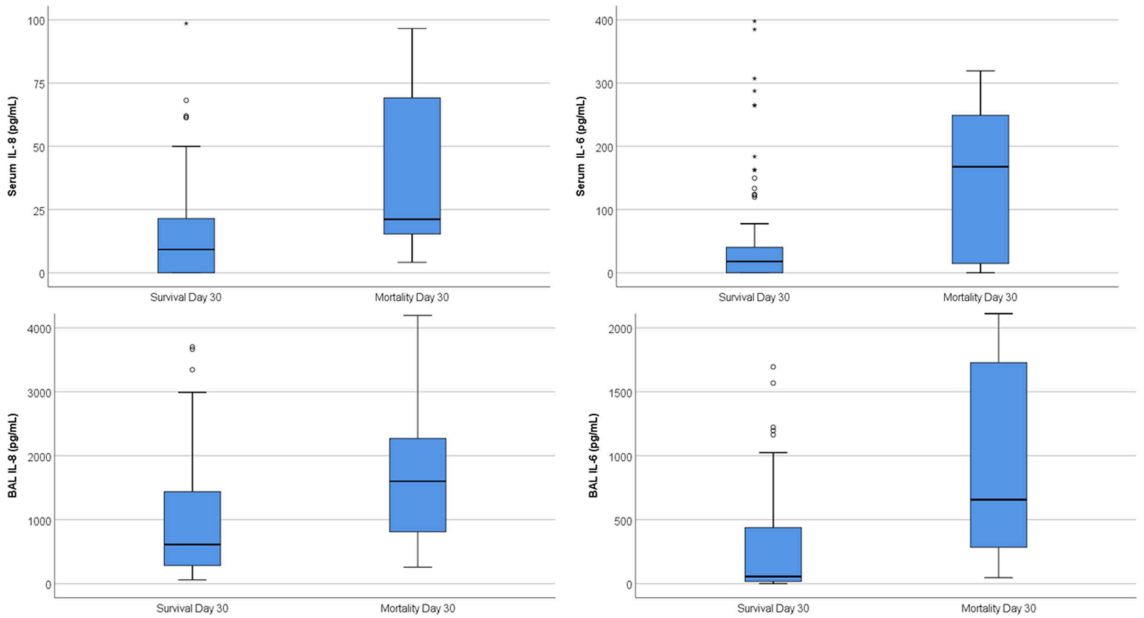

FIGURE 1 | Boxplots of same-day serum and BAL cytokines in those who survived day 30 and those who died. On the boxplot shown here, outliers are identified by different markers. "Out" values (small circle) and "far out" or as SPSS calls them "Extreme values" (marked with a star). SPSS uses a step of 1.5×IQR (Interquartile range).

FIGURE 2 | Kinetics of median interleukin-8 plasma levels from 4 days before until 4 days after bronchoscopy stratified by overall mortality categories.

in vitro opsonization of *Aspergillus fumigatus* conidia with H-ficolin, L-ficolin (17), and M-ficolin (which play essential roles in pathogen recognition and complement activation through the lectin pathway) potentiate IL-8 secretion of A549 lung epithelial cells (12, 18, 19). In line, we have previously shown that significantly better diagnostic performances were observed for serum IL-8 and also serum IL-6 when compared to established blood biomarkers (14).

It is worth noting that a number of other conditions/irritants lead to increased levels of IL-6 and IL-8 in both blood and lung environments. Studies have shown increased levels of IL-6 and IL-8 in patients with tobacco smoke exposure and/or chronic obstructive pulmonary disease (20), asthma (23), and influenza infection (24), suggesting these are relatively non-specific cytokines involved in responses to myriad insults that may be visited upon the lungs. These other conditions, including bacterial infections, may have boosted the prognostic potential of these cytokines in our high-risk cohort where IMI was suspected but only confirmed in a subset of cases, as shown in results of our sub-analyses where cytokines were also predictive of overall mortality in those with bacterial and viral infection. As a limitation, prognostic potential of

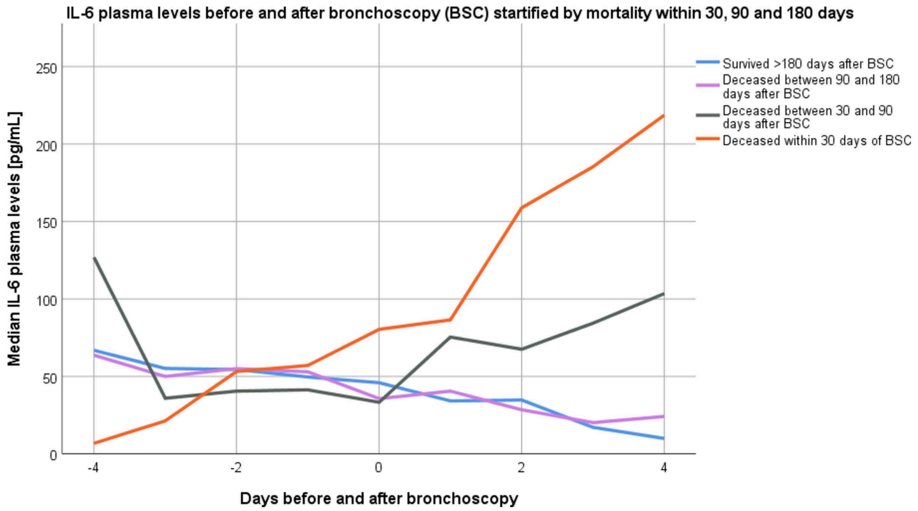

FIGURE 3 | Kinetics of median interleukin-6 plasma levels from 4 days before until 4 days after bronchoscopy stratified by overall mortality categories.

these cytokines may not be limited to patients with confirmed IMI, but may extend to patients with suspected IMI who subsequently are found to have other infections such as bacterial pneumonia. This may, however, also be considered a strength as it would allow for the use of these cytokines more broadly for treatment stratification in hematological malignancy patients with suspected pulmonary infection. While the clinical value of single measurements of cytokines may be more limited (the optimal cut-off for serum IL-8 yielded 82.4% sensitivity but only 61.8% specificity), serial measurements of these cytokines may be more promising. Overall, studies and clinical trials with larger sample sizes are needed to evaluate the prognostic potential of serial measurements of these cytokines for various subgroups of patients.

LIMITATIONS

Overall, fungal infections are rare in patients receiving anti-mold prophylaxis, with a prevalence of 2–3% (25, 26), and therefore multicenter studies are needed to confirm our findings in larger cohorts. To avoid bias introduced by multiple comparisons and confounding factors, we also had to rely on results from smaller, nested matched case-control analysis for identification of cytokines that were evaluated in the primary analyses of this cohort study. Case-control pairs in this nested analysis were matched for multiple covariates that may affect cytokine levels (27).

Additionally, autopsies were performed only in a very small subset of deceased patients and it is therefore likely that the 90- and 180-day mortality causes were not directly related to the initial reason for bronchoscopy (e.g., suspected pulmonary infection). It is still interesting that elevated levels of cytokines had predictive value for mortality so far ahead, suggesting there may be a component of specific immune dysregulation related to these cytokines playing a role in mortality in patients with underlying hematologic malignancies.

CONCLUSION

In conclusion, high blood and BAL IL-8 and IL-6 levels at the time of bronchoscopy and, in particular, increasing cytokine levels over time were predictive of mortality in a cohort of patients with underlying hematologic malignancies presenting with concern for pulmonary infection. These findings suggest it could be possible to create a treatment algorithm incorporating measurement of these cytokines at admission and throughout initial treatment for the purpose of identifying patients who warrant more aggressive treatment (e.g., combination treatment) (28, 29) when IMI is suspected in at-risk individuals.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical University Graz, Austria (EC-numbers 25-221 and 23-343). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SR, MH, JP, SE, JJ, and SH designed the study and drafted the manuscript. Data were analyzed by MH and SH. Samples were collected by SH, SE, JR, HF, AW, PN, and FP. Samples were analyzed by HS, RK, FP, JR, SH, SE, and JP. The manuscript was critically revised and important intellectual

content provided by RK, HS, HF, JR, FP, PN, and AW. The final version for publication was approved by all authors. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Revealing the Virulence Potential of Clinical and Environmental *Aspergillus fumigatus* Isolates Using Whole-Genome Sequencing

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Aspergillus fumigatus is considered a common causative agent of human fungal infections. A restricted number of virulence factors have been described, and none of them lead to a differentiation in the virulence level among different strains. Variations in the virulence phenotype depending on the isolate origin, measured as survival percentage in animal infection models, have been previously reported. In this study, we analyzed the whole-genome sequence of *A. fumigatus* isolates from clinical and environmental origins to determine their virulence genetic content. The sample included four isolates sequenced at the University Medical Center Groningen (UMCG), three clinical (two of them isolated from the same patient) and the experimental strain B5233, and the draft genomes of one reference strain, two environmental and two clinical isolates obtained from a public database. The fungal genomes were screened for the presence of virulence-related genes (VRGs) using an in-house database of 244 genes related to thermotolerance, resistance to immune responses, cell wall formation, nutrient uptake, signaling and regulation, and production of toxins and secondary metabolites and allergens. In addition, we performed a variant calling analysis to compare the isolates sequenced at the UMCG and investigated their genetic relatedness using the TRESP (Tandem Repeats located within Exons of Surface Protein coding genes) genotyping method. We neither observed a difference in the virulence genetic content between the clinical isolates causing an invasive infection and a colonizing clinical isolate nor between isolates from the clinical and environmental origin. The four novel *A. fumigatus* sequences had a different TRESP genotype and a total number of genetic variants ranging from 48,590 to 68,352. In addition, a comparative genomics analysis showed the presence of single nucleotide polymorphisms in VRGs and repetitive genetic elements located next to VRG groups, which could influence the regulation of these

genes. In conclusion, our genomic analysis revealed a high genetic diversity between environmental and clinical *A. fumigatus* isolates, as well as between clinical isolates from the same patient, indicating an infection with a mixed-population in the latter case. However, all isolates had a similar virulence genetic content, demonstrating their pathogenic potential at least at the genomic level.

Keywords: *Aspergillus fumigatus*, virulence, whole-genome sequencing, clinical and environmental isolates, gene database

INTRODUCTION

Aspergillus fumigatus is an opportunistic fungal pathogen that poses major threats to immunocompromised individuals in clinical settings. High-risk patients include neutropenic patients, hematopoietic stem cell transplant recipients, patients receiving prolonged steroid treatment, and critically-ill patients in the intensive care unit (ICU) with chronic obstructive pulmonary disease (COPD), liver cirrhosis, viral infections, or microbial sepsis (Hohl and Feldmesser, 2007; Taccone et al., 2015; Kale et al., 2017). In an individual with an impaired immune function, inhaled airborne spores of *A. fumigatus* will not be effectively eliminated and will remain in the airways, causing a range of infections that include allergic bronchopulmonary aspergillosis (ABPA), aspergilloma (chronic aspergillosis), and invasive aspergillosis (IA) (Hohl and Feldmesser, 2007; Van De Veerdonk et al., 2017). Invasive aspergillosis is the most serious infection, with a global prevalence of 250,000 cases per year and mortality rate up to 90–95% (Lin et al., 2001; Maschmeyer et al., 2007).

In addition to the increasing burden of patients with impaired immunity (Hohl and Feldmesser, 2007), another major challenge is the treatment of *Aspergillus* infections due to triazole resistance, the most commonly indicated drugs to treat these infections. Azole resistance occurs due to the presence of the point mutation L98H in the azole target *Cyp51A* and a 34-base pair (bp) tandem repeat (TR34) in its promoter region (Snelders et al., 2009). The most likely accepted cause for the development of azole resistance is the widespread azole-based fungicide use against fungal plant pathogens in agricultural practice (Snelders et al., 2009; Hagiwara et al., 2016; Meis et al., 2016).

Multiple factors drive virulence in *A. fumigatus*, and understanding the mechanisms of host adaptation and evolution of the fungus that promote the establishment of an infection, could help develop novel therapeutic strategies to treat these fungal infections (Askew, 2008). Whole-genome and transcriptome analysis have allowed the discovery and study of new components of *A. fumigatus* biology and pathogenesis. Genomic analyses have identified that *A. fumigatus* contains 8.5% of lineage-specific genes with accessory functions for carbohydrate and amino acid metabolism, transport, detoxification, or secondary metabolite biosynthesis, suggesting that this microorganism has particular genetic determinants that can facilitate an *in vivo* infection (Fedorova et al., 2008).

Virulence of *A. fumigatus* previously assessed in murine infection models using two reference strains Af293 and CEA10, showed a high variability depending on the stimuli used to compromise the immune system (Keller, 2017). However, conclusions of *A. fumigatus* pathogenicity based exclusively on observations from these two reference strains may be biased (Keller, 2017). We categorized *A. fumigatus* isolates into three different groups depending on the source of isolation: (1) environmental, e.g., obtained from decaying vegetation, air samples, or crops; (2) clinical, initially found in patient samples and; (3) experimental, which refers to isolates that were first obtained from a clinical setting, and now used as reference strains (i.e., Af293 or CEA10). Several studies have reported differences in virulence between *A. fumigatus* clinical and environmental isolates, as well as among isogenic isolates, determined by survival tests in animal infection models (Mondon et al., 1996; Alshareef and Robson, 2014; Amarsaikhan et al., 2014; Knox et al., 2016; Ballard et al., 2018). These observations highlight the need to recognize the intraspecies genotypic and phenotypic variation among *A. fumigatus* populations for determining the progression and outcome of the diseases produced by this fungus.

We hypothesized that strains from different sources could possess a different virulence genetic content. To test this hypothesis, we investigated the virulence-related genes (VRGs) of nine *A. fumigatus* isolates, represented by two experimental, five clinical, and two environmental isolates. We screened the genomes of the nine isolates using a database containing 244 *A. fumigatus* VRGs selected from studied literature. As a secondary objective, we analyzed the whole-genome sequences of three clinical isolates, two isolated from the same patient with a fatal IA infection and the other one, a colonizing isolate from another patient, and one experimental strain B5233, generated at the University Medical Center Groningen (UMCG) to identify genomic differences between them. Unlike other studies that define the virulence of *A. fumigatus* using animal infection models, this study uses genomic analysis to assess its virulence potential.

MATERIALS AND METHODS

Background of *A. fumigatus* Isolates

Aspergillus fumigatus samples evaluated in this study are summarized in **Table 1**. Four clinical isolates were included: three isolates (P1MS, P1MR, and P2CS) obtained from the UMCG, Groningen, Netherlands and the strain B5233, kindly provided by the Institute for Disease Control and Prevention of the Academy of Military Medical Sciences, Beijing, China. B5233 is a clinical isolate that demonstrated high virulence in

TABLE 1 | Characteristics of *A. fumigatus* isolates investigated in this study.

Isolate	Country	Source	Resistance	Resistance mutation	References
B5233	United States	Clinical/Experimental	Susceptible	–	Sugui et al., 2007; Jackson et al., 2009
P1MR	Netherlands	Clinical (UMCG)	Resistant	TR34/L98H	This study
P1MS	Netherlands	Clinical (UMCG)	Susceptible	-	This study
P2CS	Netherlands	Clinical (UMCG)	Susceptible	-	This study
Af293 reference	Unknown	Clinical/Experimental	Susceptible	–	Abdolrasouli et al., 2015
12-7505054	United Kingdom	Clinical	Susceptible	–	Abdolrasouli et al., 2015
08-12-12-13	Netherlands	Clinical	Resistant	TR34/L98H	Abdolrasouli et al., 2015
08-19-02-30	Netherlands	Environmental	Susceptible	–	Abdolrasouli et al., 2015
08-19-02-46	Netherlands	Environmental	Resistant	TR34/L98H	Abdolrasouli et al., 2015

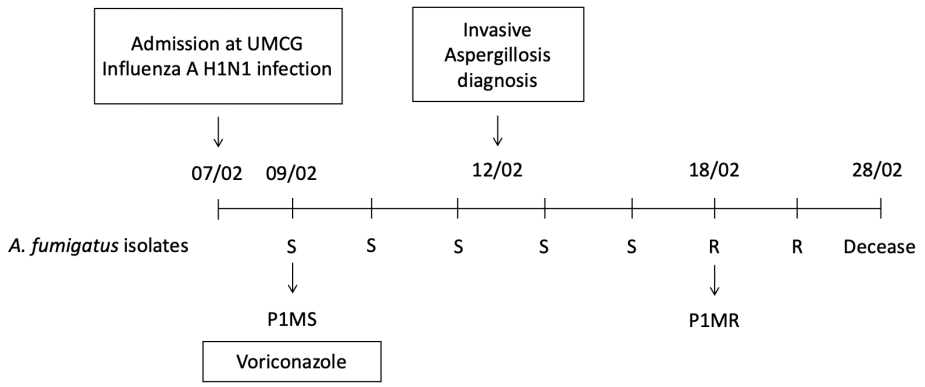

FIGURE 1 | Timeline of the influenza A (H1N1) patient staying in the hospital and the course of infection. A total of seven *Aspergillus fumigatus* isolates were obtained from sputum samples, five susceptible (S) and two resistant (R). The patient remained in the hospital for a period of 21 days until the time of death. Isolates P1MS and P1MR used in this study are indicated in the figure.

murine infection studies, and it has been used as an experimental strain in *A. fumigatus* pathogenicity studies (Sugui et al., 2007; Jackson et al., 2009). The four isolates were initially identified as *A. fumigatus* by microscopic morphological description and sequencing of the internal transcribed spacer (ITS) region using Sanger sequencing.

P1MS and P1MR, originally isolated from the sputum of the same patient at different time points during a complicated Influenza A (H1N1) virus infection, were considered as mixed-infection isolates, one susceptible and one azole-resistant isolate (**Figure 1**). This patient was diagnosed with Influenza A (H1N1) virus upon admission and had no other relevant underlying disease. Two days after admission, a positive sputum culture of *A. fumigatus* prompted the initiation of treatment with voriconazole. The patient developed IA at day 5 after admission and passed away 16 days after the diagnosis of the fungal infection. Throughout the course of the IA infection (21 days), a total of seven *A. fumigatus* isolates were recovered, the first five isolates were susceptible to azole treatment and the last two were resistant. We selected the first susceptible and the first resistant isolate to determine their genetic relatedness.

The P2CS isolate was recovered from an individual diagnosed with human immunodeficiency virus (HIV) infection and COPD. The *A. fumigatus* was cultured during a COPD exacerbation event. Doctors discarded chronic pulmonary aspergillosis after a chest imaging study, which did not show the radiological characteristics of pulmonary aspergillosis. Since no indicative symptoms of aspergillosis were identified, the patient was considered as colonized by this strain. The patient was under treatment with antiviral therapy ODEFSEY (emtricitabine/tenofovir alafenamide/rilpitvirine) and treatment for COPD with fluticason, cotrimoxazol, formeterol, and ipratropium.

In addition, the raw sequencing data of five unrelated Dutch and English *A. fumigatus* isolates of environmental and clinical origins (Abdolrasouli et al., 2015), were downloaded from the European Nucleotide Archive (ENA) and included in the study (**Table 1**).

Antifungal Susceptibility Testing

The *in vitro* susceptibility of isolates B5233 and P1MS to triazole antifungal drugs was determined using an E-test (AB BIODISK, Solna, Sweden), the agar-based gradient technique for quantitative antifungal susceptibility. The agar-based method VIPcheck^{TM} test (Nijmegen, Netherlands) was used for isolate P2CS, and the susceptibility of P1MR was determined with the *in vitro* broth microdilution reference method from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2019).

DNA Isolation

Isolates were grown on Potato Dextrose Agar for 7 days at 35°C. The DNA extraction was performed using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with some modifications in the initial steps. The initial fungal starting material was obtained using a pre-wetted sterile swab rubbed against the sporulating colony that was dissolved in 700 μl sterile saline solution. The suspension was centrifuged at 10,000 rcf for 4 min. The supernatant was discarded, and the pellet was resuspended in 300 μl of Power Bead solution. This suspension was added to a second pellet of the same sample. The final concentrated suspension was transferred to a Pathogen Lysis Tube L containing beads (Qiagen, Hilden, Germany), 50 μl of solution SL, and 200 μl of sterile saline solution for homogenization. Disruption was carried out in a Tissue Homogenizer Precellys 24 (Bertin, Montigny-le-Bretonneux, France), set to three times at 5,000 rpm for 30 s, and separated by 30 s. The disruption preps were heated to 65°C, as suggested in the Troubleshooting Guide of the protocol, to increase the final DNA yield.

Library Preparation and Whole-Genome Sequencing

The procedure was performed according to the manufacturer's protocol (Illumina, San Diego, CA, United States). The fungal genomic DNA (gDNA; 1 ng) of each specimen was used as input DNA for library preparation with NexteraXT DNA Library Prep Kit. Library quality was determined by measuring the fragment size on a 2200 TapeStation System with D5000 Screen tape (Agilent Technologies) and quantified with Qubit 2.0 Fluorometer using Qubit dsDNA HS Assay Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, United States). NexteraXT libraries were denatured and diluted to the required molarity for the Illumina sequencing platform and then, two pools were made containing each two libraries. Whole-genome sequencing was performed in two separate runs using the MiSeq Reagent Kit v2 500-cycles Paired-End on a MiSeq Sequencer (Illumina).

Quality Control and *de novo* Assembly

The raw sequencing reads were quality trimmed using the CLC Genomics Workbench software v10.1.1 with default settings, except for the modification where 'trim using quality scores was set to 0.01.' The quality of the nine *A. fumigatus* genome assemblies is shown in **Supplementary Table 1**. *De novo* assembly produced acceptable results that surpassed >100 coverage with >90% of reads used.

Identification of Virulence-Related Genes

We considered the review on genes and molecules involved in IA by Abad et al. (2010), the online gene database AspGD[1], and bioactive secondary metabolite genes encoding Biosynthetic Gene Clusters (BGCs) 3, 5, 6, 14, 15, and 25, as part of the pathogenic arsenal of this fungus (Bignell et al., 2016; Kjaerbølling et al., 2018) to build a comprehensive database. The 244 VRGs included in the database could be categorized into seven groups according to their involvement in processes, such as thermotolerance, resistance to immune responses, cell wall formation, nutrient uptake, signaling and regulation, and production of toxins and secondary metabolites and allergens (**Supplementary Table 2**). The *de novo* assemblies of the clinical UMCG isolates and strain B5233 were screened with the ABRicate v0.3 software tool[2] to detect the presence or absence of VRGs included in the database. The thresholds were set to >90% coverage and >90% identity to determine the presence of a VRG.

TRESP Genotyping

This method is based on hypervariable tandem repeats located within exons of surface protein coding genes (TRESP) related to cell wall or plasma membrane proteins (Garcia-Rubio et al., 2016). The allele sequence repeats of three TRESP targets, an MP-2 antigenic galactomannan protein (MP2), a hypothetical protein with a CFEM (common in several fungal extracellular membrane proteins) domain (CFEM), and a cell surface protein A (CSP) are combined to assign a specific genotype (Garcia-Rubio et al., 2016). The previously described allele repeats of these proteins were used to Create a Task Template by Allele Libraries in SeqSphere + software v5.1.0 (Ridom GmbH, Münster, Germany) with import option: use as reference sequence 'best matching allele' that enabled a dynamic reference sequence. The assembled genomes were imported into SeqSphere + and the specific target repetitive sequences of each protein were analyzed for each UMCG isolate using the 'find in sequence' tool to identify the specific allele combination.

Comparative Genomics

Genome assemblies of UMCG isolates and B5233 were aligned using blast + v2.6 (Camacho et al., 2009), and reads were mapped to the eight reference chromosomal genomes of Af293 (Accession No. NC_007194 -NC_007201) using bowtie2 v2.2.5 (Langmead and Salzberg, 2012). For each contig, local alignment coordinates were extended to their whole length by using the highest bitscore with an in-house script. Mean coverage was calculated every 5 kb using bedtools v2.17 (Quinlan and Hall, 2010). The location of VRGs was determined by local alignment, and GC percentage was calculated every 100 bp with a script[3]. Location and frame of coding sequences were extracted from the reference sequence GenBank files. All gathered information was represented in a circular image using circos v0.69-3 (Krzywinski et al., 2009).

Identification of Genetic Variants

The variant analysis was performed for the three UMCG isolates, B5233 strain, and two Dutch environmental isolates named 08-19-02-30 and 08-19-02-46. Variants were called against the reference genome *A. fumigatus* Af293 (release 37, FungiDB) using the web-based platform EuPathDB Galaxy

[1]http://www.aspgd.org/

[2]https://github.com/tseemann/abricate

[3]https://github.com/DamienFr/GC-content-in-sliding-window-

Site[4] (Giardine et al., 2005). The quality control of the raw reads was performed with FastQC (version 0.11.3, Babraham Institute) and trimmed with Sickle (Galaxy version 070113). Trimmed-reads were aligned with the reference using Bowtie2 (Align version 2.1.0 64) (Langmead et al., 2009) and the 'very sensitive' alignment default setting. The BAM files were sorted with SAMtools, and variant calling was performed with Freebayes (v0.9.21-19-gc003c1e) and SAMtools (Li et al., 2009). The resulting variants were annotated using SnpEff to predict the impact of a variant on the gene function, classifying them into different categories: high, moderate, low, and modifier[5] (Cingolani et al., 2012). High impact variants are predicted to have a disruptive effect on the protein (e.g., frameshift variants, inversion), moderate impact variants could change protein effectiveness (e.g., missense variant, in-frame deletion), low impact variants are not expected to have a significant impact on protein function (e.g., synonymous variant), and finally, modifier variants are non-coding changes where predictions are difficult, or there is no evidence of impact (e.g., exon variant, downstream gene variant). SnpSift was used to extract the variants with moderate and high impact by filtering the resulting variant call format (VCF) files from SnpEff (**Supplementary Material 5–10**).

In addition, identification of single nucleotide polymorphisms (SNPs) present in VRGs of UMCG isolates and B5233 strain was performed using CLC Genomics Workbench software v11.0.1. For this purpose, trimmed-reads of each genome were mapped to a concatenated sequence consisting of 244 VRG genes (**Supplementary Table 2**). The SNPs were called with a minimum read coverage of 10 and with a minimum frequency of 90%. The VRG sequences used to create the concatenated sequence belonged to the reference *A. fumigatus* Af293.

Snippy v. 4.3.5[6] was used to determine the number of variants between P1MS and P1MR isolates. The trimmed-reads of P1MR were aligned to the assembly of P1MS for variant calling. In this case, the P1MS draft genome assembly, which is used as the reference, is not annotated, and therefore, a functional prediction of the determined variants was not possible. Accordingly, we only presented a quantitative analysis of the latter.

RESULTS

Screening of Virulence-Related Genes

We screened the genome sequences of nine *A. fumigatus* isolates (**Table 1**) for the presence of particular VRGs using our in-house database (**Supplementary Table 2**). We identified the presence of all 244 VRGs (>90% coverage and >90% identity) in the genome of seven isolates: P1MR, P1MS, P2CS, Af293, 12-7505054, 08-19-02-30, and 08-19-02-46. In addition, 243 genes were present in the genomes of B5233 and 08-12-12-13, and both isolates lacked the *Afu5g12720* gene. This gene codes for a putative ABC transporter and is a member of the BGC17, consisting of 10 genes (Bignell et al., 2016). The product of this BGC is a non-ribosomal peptide synthetase, thought to have a structural function (O'Hanlon et al., 2011). However, no clear link between this ABC transporter and the function of this peptide has been described before. Therefore, it is unknown how its absence could affect the overall function of this cluster and its specific role in mediating virulence.

TRESP Genotyping

We used the TRESP genotyping method to determine if the *A. fumigatus* isolates were genetically related. This is especially interesting in the two isolates obtained at different time points from the same patient suffering from an influenza A (H1N1) infection with IA (**Figure 1**). We wondered whether the susceptible P1MS isolate and the resistant P1MR isolate with 9 days of isolation difference were isogenic, and whether the resistant phenotype developed after azole treatment. The UMCG isolates and B5233 strain presented different allelic combinations, and thus, different TRESP genotypes: P1MS and P1MR having t03m1.1c08A and t11m1.1c09 TRESP genotypes, respectively. In this study, CSP alleles best differentiated the isolates (**Table 2**).

Comparative Genomics

We compared the genomes of our UMCG isolates and B5233 strain with the *A. fumigatus* Af293 chromosomes. The comparison of the genomic sequences of the eight chromosomes is shown in **Figure 2**; the researched VRGs locations are highlighted in yellow. We observed small deletions (100 kbp) at the end of chromosomes 5 and 6, and large deletions (>300 kbp) at the beginning of chromosome 1 and at the end of chromosome 7. Multiple small deletions and large-scale deletions in *A. fumigatus* genomes have been reported, and particularly, the large-scale deletions were previously described in chromosome 1 (Abdolrasouli et al., 2015; Garcia-Rubio et al., 2018) and chromosome 7 (Garcia-Rubio et al., 2018). A region with a high dissimilarly, ranging from 1,698 to 2,058 kbp, compared to the reference Af293 is observed in chromosome 7 for all the isolates, except for P2CS that had a certain degree of similarity (**Figure 2**). Sequence gaps with no assigned CDS represent: (i) putative centromeres in all chromosomes (indicated by a red line in **Figure 2**), and (ii) a region of ribosomal DNA in the chromosome 4 (indicated by a dark blue line in **Figure 2**) (Fedorova et al., 2008). We observed repeat-rich sequences in chromosomes 1, 2, 4, 6, and 8, represented by the alignment of many small contigs that coincides with a low GC content (**Figure 2**). In the case

TABLE 2 | TRESP genotype based on repetitive sequences in the exons of surface proteins CSP, MP2, and CFEM.

Sample	Allele CSP	Allele MP2	Allele CFEM	TRESP genotype
B5233	t02	M1.2	c09	t02m1.1c09
P1MR	t11	M1.1	c09	t11m1.1c09
P1MS	t03	M1.1	c08A	t03m1.1c08A
P2CS	t02	M1.1	c19	t02m1.1c19

[4]https://eupathdb.globusgenomics.org/

[5]http://snpeff.sourceforge.net/SnpEff_manual.html

[6]https://github.com/tseemann/snippy

of chromosome 4, a group of VRGs appears to be flanked by these repetitive regions on both sides, whereas some groups are only flanked on one side as depicted in chromosomes 6 and 8 (**Figure 2**).

Genomic Variability Among the Fungal Genomes

Variant calling using the *A. fumigatus* Af293 genome as reference identified a total number of 68,352; 48,590; 56,362; and 56,422

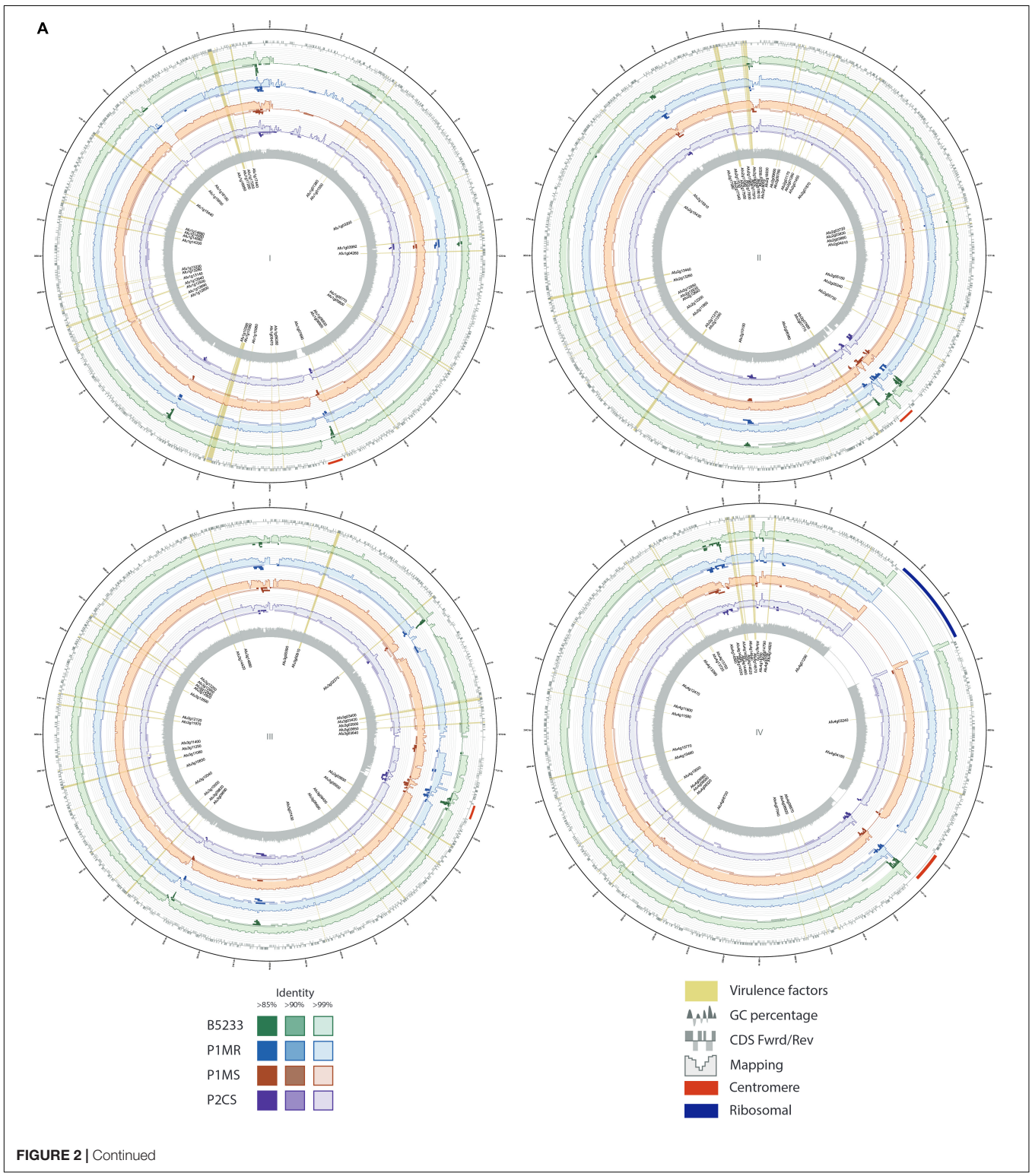

FIGURE 2 | Continued

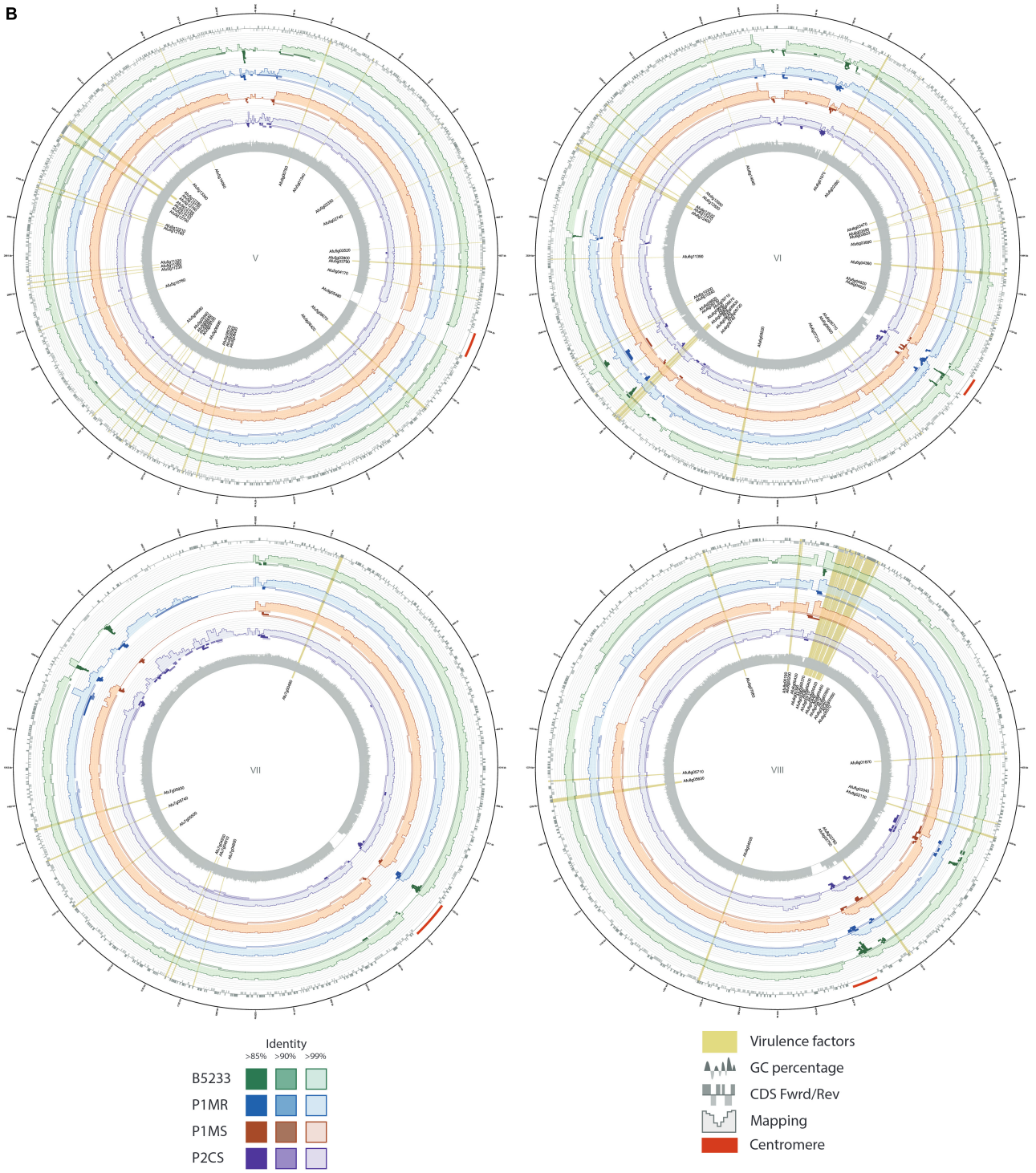

FIGURE 2 | Graphical representation of assemblies and reads of B5233 (green), P1MR (blue), P1MS (orange), and P2CS (purple) isolates aligned to Af293 reference chromosomes: I, II, III, IV **(A)**; V, VI, VII, VIII **(B)**. Outer track indicates all CDS in forward (dark gray) or reverse (light gray) strand. Two different tracks are represented per isolate: one corresponding to the mapping coverage and another one corresponding to contig alignment (minimum ID 85%). The complete contigs are represented with transparency in accordance to the local alignment identity. Genes related to virulence are highlighted in yellow with its names in the innermost track. GC% is represented every 100 bp. Red lines indicate putative centromeres and the dark blue line (chromosome 4) represents the ribosomal DNA.

variants in the genome of B5233, P1MS, P1MR, and P2CS isolates, respectively (**Table 3**). High and moderate impact variants were retrieved, and their predicted effect is displayed in **Supplementary Table 3**. Among the predicted moderate- and high-impact variations, a high number of missense variants ranging from 9,804 to 12,067 were identified that could affect the gene function (**Supplementary Table 3**). The SNP analysis in VRGs with respect to the reference Af293 strain revealed the presence of SNPs in the range of 1,015–1,122 in all the analyzed isolates (**Table 3**). Examples of some variants present in the VRGs are listed in **Table 4**, and a more detailed description is given in **Supplementary Table 4**. We did not observe any distinct pattern of variant distribution among the VRGs, and thus, we could not assign a particular variant profile based on the origin of the isolate (**Table 4**). Instead, we observed some cases where all the isolates had the same SNPs in the same gene as demonstrated for *thtA, sidC*, and *msdS* genes. Genes associated with resistance to the immune response, such as *rodB*, *cat1*, and *afpmt2* had only one or no variants, suggesting that they are highly conserved genes. The *gliZ* gene, required for the regulation of gliotoxin and the *gli* cluster, as well as the *sidC* gene, with an essential role in iron acquisition, are examples of genes with different variants in the studied isolates.

Additionally, we performed a comparative analysis between P1MS and P1MR isolates from the same patient and detected 45,335 variants, corresponding to 38,319 SNPs; 868 multiple nucleotide polymorphisms (MNPs); 1,768 insertions; 1,842 deletions; and 2,538 complex mutations (a combination of SNPs and MNPs).

DISCUSSION

Aspergillus fumigatus is a major fungal pathogen capable of causing chronic and deadly invasive infections. Here, we performed a genomic analysis to investigate the virulence potential of this pathogen at the genomic level. We hypothesized that *A. fumigatus* isolates recovered from a patient who died after infection with influenza A (H1N1) and IA, and an isolate from a patient with HIV and COPD with no reported *Aspergillus* infection would reveal a distinct virulence genomic content. In addition to our clinical isolates, we studied the known virulent *A. fumigatus* experimental strain B5233 and the genomes of five unrelated isolates available in a public database, their source of isolation being different (**Table 1**).

TABLE 3 | Variant analysis of the novel *A. fumigatus* isolates against reference *A. fumigatus* Af293.

Isolates	SnpEff	SnpSift filter		SNPs present in the virulence genes (CLC Genomics Workbench)
	Total number of variants	Moderate	High	
B5233	68,352	12,085	884	1122
P1MS	48,590	10,109	752	1107
P1MR	56,362	11,718	870	1015
P2CS	56,422	12,085	884	1158

Our analysis identified 244 VRGs in all tested *A. fumigatus* isolates, with the exception of *Afu5g12720* gene in B5233 and 08-12-12-13 genomes, indicating that all the studied isolates had the genetic information for virulence. These results suggest that differences in the *A. fumigatus* virulence capacity may not be determined by the presence or absence of virulence factors at the genomic level. This finding is in concordance with the development of an *Aspergillus* infection depending primarily on the alteration of the host immune status. Moreover, the high variability in the studied *A. fumigatus* genomes reflects the enormous capacity of the fungus to adapt to different environments.

Amongst the 244 VRGs included in our in-house database, the *Afu5g12720* gene was the only gene that was undetected in B5233 and 08-12-12-13 isolates. This gene was reported to be absent in 21 out of 66 *A. fumigatus* samples in a population genomics study that investigated the genomic variation of secondary metabolites in this species (Lind et al., 2017). This gene is a member of the BGC17, and its absence could have a functional impact on the synthesis of the final product of this cluster, a non-ribosomal peptide synthetase, which is thought to have a structural function (O'Hanlon et al., 2011). The *Afu5g12720*, coding for an ABC transporter is located in the BGC17 along with other nine genes (Bignell et al., 2016) and was absent in B5233, an experimental strain that has been described as highly virulent. It would be interesting to further study the link between the lack of this gene and a possible increase in virulence, since disruption of another gene member of BGC17, *pes3*, resulted in a hypervirulent strain (O'Hanlon et al., 2011).

The comparative genomic analysis provided additional information about changes in the genome structure of our isolates. We observed an absence of segments at the beginning of chromosome 1 and at the end of chromosomes 5, 6, and 7 in isolates B5233, P1MS, and P1MR when compared with the reference strain Af293. Fedorova et al. (2008) described these segments as subtelomeric regions enriched for the presence of pseudogenes, transposons, and other repetitive elements. Previous reports have suggested that these genes have most likely evolved from big duplication and diversification events and not from horizontal gene transfer (Fedorova et al., 2008). Likely, these segments are insertion-prone regions that contribute to the diversification of the species.

The nucleotide variant analysis of four isolates sequenced at the UMCG identified 48,590 to 68,352 variations compared with the reference strain Af293. This range is similar to the previously reported genetic diversity for *A. fumigatus* determined in 95 sequences, ranging from 36,000 to 72,000 SNPs (Knox et al., 2016). The large number of identified variants and differences in the genome structure displays a broad genetic diversity in the studied isolates. This diversity is hypothesized to directly influence the virulence of the fungus by allowing adaptation to the host environment, the evasion of the host immune system, and the acquisition of antifungal resistance (Rizzetto et al., 2013; Hagiwara et al., 2014; Verweij et al., 2016; Ballard et al., 2018). The presence of SNPs in the VRGs of the clinical isolates, particularly those

TABLE 4 | Examples of shared and unique moderate and high impact variants in genes associated to thermotolerance, resistance to the immune response, cell wall formation, nutrient uptake, and production of toxins and secondary metabolites and allergens.

Gene	Conserved in all	Per strain					
		B5233	P1MR	P1MS	P2CS	08-19-02-30	08-19-02-46
thtA	c.3698T > C c.3458T > C	c.3277C > T	–	c.2828C > T c.2188C > T c.1010G > T	c.3277C > T c.-598G > A	c.272C > A c.2128C > T HIGH stop gained p.Arg710*	–
pmt1	–	–	c.442G > A	–	–	–	–
rodB	–	–	–	–	–	–	–
cat1	–	–	–	–	–	–	–
catA	–	–	c.1385G > A	c.1385G > A c.982G > A	–	–	–
afpmt2	–	–	–	–	–	–	–
laeA	–	–	–	c.189G > A HIGH stop codon gained p.Trp63* c.400C > T	–	–	–
gliZ	–	c.1177C > G c.425_427delCAA disruptive_in frame_ deletion p.Thr142del	c.79A > G c.99_101dupTGC c.388A > G c.405_406insACAACAACAACA c.406_409delGCAGinsACAA c.464T > G c.612C > T c.718T > G c.1087T > C	c.388A > G c.464T > G c.1087T > C c.397_409delGCAGCAGCAGCAGinsACAACAACAACAAAAACAA missense_variant&disruptive_inframe_insertion	c.388A > G c.464T > G c.1087T > C c.99_101dupTGC disruptive_inframe_insertion c.411_412insGCAACAACA c.412A > G	c.388A > G c.464T > G c.397_409delGCAGCAGCAGCAGinsACAACAACAACAAAAAA missense_variant&disruptive_inframe_insertion c.1338_1340delCTC disruptive_inframe_deletion c.1435A > G	c.1177C > G c.412A > G c.406_411dupGCAGCA c.415A > G c.1404A > C
msdS	c.295T > C HIGH stop_lost p.Ter99Glnext*?	c.328C > G c.208T > A		c.328C > G c.208T > A	c.328C > G c.208T > A		c.328C > G c.208T > A c.1043C > T
sidC	c.3391A > G c.9598G > A c.9727T > C c.11935T > G c.14222G > A	c.577A > G c.1569C > G c.2311G > A c.3820A > G c.7174A > G c.11326A > G	c.577A > G c.1569C > G c.2311G > A c.3820A > G c.7174A > G c.3401C > T c.13019A > T	c.751C > G c.13019A > T	c.878C > T c.11341C > T c.11935T > G	c.1781T > C c.4771C > A c.5798T > C	c.577A > G c.1569C > G c.2311G > A c.3820A > G c.7174A > G c.9769C > A c.13067A > C

* = *Stop codon gained.*

predicting high impact variations, could be of major influence in the virulence of these isolates. However, we could not link the presence of nucleotide changes in VRGs with a specific origin of isolation. In addition, some repetitive elements were located on the sides of some groups of VRGs, as exemplified by chromosomes 6 and 8. These repetitive sequences could play a role in the expression of these genes since they are recognized to shape fungal genomes (Muszewska et al., 2017). Follow-up studies using RNA sequencing could help elucidate the expression of these virulence genes as well as determine the impact of genomic variations on expression levels. Subsequent infection model studies in animals could be used to correlate these genomic variations and changes with specific pathogenic phenotypes.

The genome sequence of isolates P1MS and P1MR differed by 45,335 variants, and they had different TRESP genotypes, indicating the presence of different *A. fumigatus* isolates with different azole susceptibility profiles in the same patient. It is unlikely that the susceptible isolate would have been able to mutate and acquire azole resistance in a period of 9 days since the median time of development of azole resistance has been reported to be 4 months (Camps et al., 2012). Moreover, the emergence of the resistant phenotype within the host is observed in chronic infections, whereas the acquisition of resistance during IA continues to be unreported (Verweij et al., 2016). However, our current approach cannot determine if the resistant isolate co-existed with the susceptible population since the beginning, or if the resistant isolate was newly acquired during the hospital stay.

In a similar case of post-influenza aspergillosis, four *A. fumigatus* isolates were obtained from a patient that received an allogeneic stem cell transplant and developed IA after the influenza virus infection, which was initially treated with voriconazole (Talento et al., 2018). The first three isolates were susceptible to azole treatment, while the last one exhibited triazole resistance. The resistant isolate differed from the initial isolates as confirmed by STR*Af* microsatellite genotyping (Talento et al., 2018).

The most plausible hypothesis is that the resistant *A. fumigatus* isolate, both in our study and the post-influenza study (Talento et al., 2018), was of environmental origin and that it co-existed with the susceptible isolates in a mixed population that was not detected during the first sampling. Treatment with voriconazole most probably eradicated the initial susceptible strain, and through selective pressure, allowed the resistant *A. fumigatus* strains to persist in the patient's airways. The possibility of an initial mixed population led to a change in the method of *A. fumigatus* isolation at the diagnostics laboratory at the UMCG; antifungal susceptibility testing is now applied to at least five colonies obtained from a single respiratory sample. Previous studies have reported that influenza infections alter the host immune response, favoring an *Aspergillus* co-infection (Lee et al., 2011; Ghoneim et al., 2013; Crum-Cianflone, 2016). Recently, influenza virus infection has been described as a clear independent risk factor for invasive pulmonary aspergillosis. Therefore, extreme care is advised for patients admitted into the ICU with severe influenza virus infection (Schauwvlieghe et al., 2018).

In this study, TRESP genotyping indicated that the isolates were genetically unrelated. This genotyping method was easy and accessible, and only required the whole-genome sequence of the isolates in contrast to other traditional typing methods, such as MLST, with a lower discriminatory power (Vanhee et al., 2009), the laborious microsatellite determination method (STR*Af*) (Klaassen and Osherov, 2007), or the novel whole-genome SNP-based typing method, which is highly dependent on the variant calling parameters and selection of a genetically close reference strain (Garcia-Rubio et al., 2018).

Our results are in agreement with the hypothesis that the basis of *A. fumigatus* virulence is provided by the evolution of the distinct mechanisms of stress resistance, but lacks dedicated virulence factors, in contrast to bacterial pathogens (Mccormick et al., 2010; Rizzetto et al., 2013). To define the virulence of an *A. fumigatus* isolate, many researchers have characterized different aspects of the fungus, such as the differences in the colonial and spore color phenotype (Rizzetto et al., 2013), the strain-dependent immunomodulatory properties induced in the host (Rizzetto et al., 2013), the clinical or environmental source of the isolate (Mondon et al., 1996; Rizzetto et al., 2013; Kowalski et al., 2016), the ability to adapt and grow in stressful conditions such as low oxygen microenvironments where hypoxia fitness strongly correlated with an increase in virulence (Kowalski et al., 2016), and the ability of the fungus to adjust its gene expression to survive in different immunosuppressive conditions inside the host (Kale et al., 2017). Further research on the virulence of this microorganism should take into consideration all these aspects to determine their infectivity. The results can be used to explore the link between the virulent phenotype and genotype to understand the mechanisms of infection of this pathogen.

This study has some limitations to be considered. First, the number of isolates was small, although three different *A. fumigatus* population sources (clinical, environmental, and experimental) were included. Nevertheless, our findings should be investigated in a larger population to fully corroborate the observation that all members of this species are potentially pathogenic at the genetic level. Second, we included 244 genes in our in-house database based on the current knowledge of *Aspergillus* virulence, but we do not rule out the possibility that other genes may be related to virulence.

CONCLUSION

We developed an in-house database with 244 VRGs and detected all of them, except *Afu5g12720,* in the whole-genome sequence of five clinical, two environmental, and two experimental *A. fumigatus* isolates. We did not observe any association between a virulence genetic content and an isolate of specific origin. Moreover, a broad genomic variability and the convenient location of transposable elements that are known to shape the genome reflects the adaptability of *A. fumigatus,* which challenges the development of effective treatments and specific diagnostic tools. Understanding the expression mechanisms

of the VRGs may ultimately explain the regulation of the virulence of *Aspergillus* and help improve the handling of *A. fumigatus* infections.

DATA AVAILABILITY

The sequence raw reads generated in this study have been submitted to the European Nucleotide Archive under project accession number PRJEB28819. Variant analysis results are available as **Supplementary Material**.

ETHICS STATEMENT

The fungal isolates used for the present analyses were collected in the course of routine diagnostics and infection prevention control. Oral consent for the use of such clinical samples for research purposes is routinely obtained upon patient admission to the UMCG, in accordance with the guidelines of the Medical Ethics Committee of the University Medical Center Groningen. All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

AUTHOR CONTRIBUTIONS

FP-B, SG, MC, and CO designed the experimental set-up. SG, MC, JR, and MS supervised the study. FP-B, ER, and MP performed the experiments. FP-B, SG, MC, and PS-C analyzed the data. SG, JR, MS, LH, and MC provided constructive criticism on the writing. FP-B wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01970/full#supplementary-material

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TNF-α-Producing *Cryptococcus neoformans* Exerts Protective Effects on Host Defenses in Murine Pulmonary Cryptococcosis

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Tumor necrosis factor alpha (TNF-α) plays a critical role in the control of cryptococcal infection, and its insufficiency promotes cryptococcal persistence. To explore the therapeutic potential of TNF-α supplementation as a booster of host anti-cryptococcal responses, we engineered a *C. neoformans* strain expressing murine TNF-α. Using a murine model of pulmonary cryptococcosis, we demonstrated that TNF-α-producing *C. neoformans* strain enhances protective elements of host response including preferential T-cell accumulation and improved Th1/Th2 cytokine balance, diminished pulmonary eosinophilia and alternative activation of lung macrophages at the adaptive phase of infection compared to wild type strain-infected mice. Furthermore, TNF-α expression by *C. neoformans* enhanced the fungicidal activity of macrophages *in vitro*. Finally, mice infected with the TNF-α-producing *C. neoformans* strain showed improved fungal control and considerably prolonged survival compared to wild type strain-infected mice, but could not induce sterilizing immunity. Taken together, our results support that TNF-α expression by an engineered *C. neoformans* strain while insufficient to drive complete immune protection, strongly enhanced protective responses during primary cryptococcal infection.

Keywords: cryptococcosis, TNF-α, host defense, TNF-α-producing *C. neoformans*, Th1/Th2 balance

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INTRODUCTION

The fungal pathogen *Cryptococcus neoformans* causes substantial morbidity and mortality worldwide, accounting for an estimated 220,000 cases of cryptococcal meningoencephalitis and 180,000 deaths among people with HIV/AIDS each year (1–4). The current treatment options for cryptococcal infections are limited and often unsuccessful due to the high toxicity and the poor blood-brain barrier permeability of the antifungal drugs (5, 6). Acute mortality for cryptococcal infections is up to 30%, and many surviving patients may develop permanent disabilities (7–9).

Proper containment of *C. neoformans* by host requires the development of optimal innate and adaptive immune responses (2). Murine models have shown that protective anti-cryptococcal immunity depends on the generation of strong Th1/Th17 T-cell responses, which in turn, promote classical activation of macrophages and effective containment of *C. neoformans*. In contrast, Th2 response facilitates eosinophil recruitment and alternative activation of macrophages, which promote intracellular survival and growth of *C. neoformans* during infection (2, 10–13). Apart from the effector functions during the adaptive response, macrophages, and dendritic cells (DC) play active roles in the early/innate defenses. They ingest *C. neoformans*, providing first-line of anti-fungal containment in the infected lungs. Through fungal antigen processing, presentation, and concurrent inflammatory cytokine production, they initiate and polarize the development of T-cell responses (10, 14).

Tumor necrosis factor alpha (TNF-α), a major player during innate and adaptive responses, has been shown to play critical roles in host defense against *C. neoformans* infection. Early TNF-α production in a murine model infected with a moderately virulent *C. neoformans* strain 52D is required for the development of protective Th1/Th17 immune responses and subsequent fungal containment (15–17). Recent studies showed that early TNF-α induction contributes to DC classical activation, followed by the generation of protective adaptive responses during the efferent phase of *C. neoformans* infection (17, 18). Furthermore, the enhanced risks for cryptococcal infection in individuals undergoing anti-TNF-α therapies for sepsis, cancer, and autoimmune disorders provided strong evidence that this cytokine also plays an important role in mediating host defense against *C. neoformans* infection in human (18–20). However, highly virulent *C. neoformans* strains are endowed with a capsule that can attenuate host production of TNF-α (21, 22). During infection, mice infected with a highly virulent *C. neoformans* strain show significantly lower TNF-α expression in the lungs compared to mice infected with less virulent *C. neoformans* strain (23). Thus, highly virulent *C. neoformans* could evade host anti-cryptococcal immunity by inducing minimal TNF-α response.

Considering this broad significance of TNF-α and the inhibitory effect of highly virulent *C. neoformans* on its production, we hypothesized that therapeutic delivery of TNF-α could be a mean for augmenting host immune defenses against *C. neoformans* infections. Studies have shown that *C. neoformans* strain constructed to constitutively produce and secrete low of murine IFN-γ successfully induced protection against primary and second pulmonary challenges with a pathogenic *C. neoformans* strain H99 (24). In this study, we generated analogous *C. neoformans* strain engineered to express murine TNF-α with a goal to stimulate a protective response against cryptococcal infection. These studies have provided evidence that TNF-α expression by highly virulent *C. neoformans* strain can enhance protective responses and suggest that adjuvant TNF-α therapy may represent a viable approach supporting treatment of *C. neoformans* infection.

MATERIALS AND METHODS

Ethics Statement

These studies were performed in compliance with the protocols approved by the institutional R&D, animal studies, and research safety committees of the VA Ann Arbor Health System. Ann Arbor VA Animal Studies Committee approved these studies (protocol number 0512-025) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All manipulations involving live mice were performed under general anesthesia, and endpoint criteria were used for survival experiments to humanely euthanize moribund animals. All efforts were made to ensure proper care for the animals and to minimize suffering.

Mice

Female Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed under specific-pathogen-free (SPF) conditions at the Ann Arbor Veterans Affairs Medical Center. Animals were 8–12 weeks old at the time of infection. Mice were humanely euthanized using CO_2 inhalation at the time of data collection.

C. neoformans

For H99-α construction, an expression construct containing cryptococcal *ACTIN* promoter and murine TNF-α cDNA sequences was transformed into *C. neoformans* serotype A strain H99 by a biolistic method as described previously (25, 26). For inoculation, *C. neoformans* H99-α and wild-type (WT) H99 strain were grown for 4 days at 37°C using Sabouraud dextrose broth (1% Neopeptone, 2% dextrose; Difco, Detroit, MI, USA). The cultures were centrifuged and washed with non-pyrogenic saline (Travenol, Deerfield, IL, USA). Cells were counted via hemocytometer and diluted to 3.3×10^5 yeast/ml in sterile non-pyrogenic saline. Serial dilutions of the *C. neoformans* suspension were plated on Sabouraud dextrose agar (SDA) to confirm the number of viable fungi in the inoculum.

Transformation of *C. neoformans* With a Murine TNF-α Construct

In order to avoid the disruption of exogenous DNA constructs on adjacent genes, we remodeled the plasmid vector (pCH233) to facilitate the integration of the TNF-α cassette into a small gene-free region ("safe haven") as previously described (27). The "safe haven" region was PCR amplified from *C. neoformans* genomic DNA as two fragments using primers ZF0001&ZF0002 and ZF0003&ZF0004 and the nourseothricin resistance marker NAT was amplified from pCH233 using primers ZF0005&ZF0006 to give a 1.9 kb product. The three fragments were joined to generate a 3.5 kb product via overlap PCR using primers ZF0001 and ZF0004, purified, and then infused with purified vector sequence (amplified from the plasmid pCH233 by PCR using primers ZF0011&ZF0012 containing SfiI sites on both 5′ and 3′ flanking regions) by pEASY-Uni Seamless Cloning and Assembly Kit (Transgen Biotech). The new vector pCH235 was transformed into *E. coli*, and confirmed by diagnostic PCR (using primers ZF0001 and ZF0005) and sequencing.

The *ACTIN* promoter region and TRP1 terminator region were amplified from the vector pCH233 by PCR using High Fidelity DNA Polymerase PrimeSTAR (Takara) using primers ZF0007&ZF0008 and ZF0009&ZF0010, respectively. The whole encoding region was synthesized by Thermo Fisher Scientific, including the signal sequence of the phospholipase B (PLB) gene from genomic DNA derived from *C. neoformans* strain H99 and Murine TNF-α cDNA (soluble form, GenBank No. AK155964.1). Above fragments were then infused into a murine TNF-α construct (1.8 kb, **Figure 1A**) by overlap PCR using primers ZF0007 and ZF0010 containing each HindIII site on both upstream and downstream flanking regions. The TNF-α construct was purified and then infused with linearized vector pCH235 (digested with restriction endonuclease HindIII). The new plasmid pCH-TNFA was digested with SfiI to generate a 5.3 kb product (TNF-α integration cassette) containing the safe haven, murine TNF-α construct and *NAT* gene. The final product was purified and then transformed into the *C. neoformans* genome via biolistic delivery system as previously described (26). Transformants were selected on YPD media plus 100 μg/ml nourseothricin (clonNAT; Werner Bioagents, Jena, Germany), and confirmed by Southern blot, colony PCR and real-time PCR. Culture supernatant of TNF-α secreting and WT *C. neoformans* strains were evaluated for TNF-α protein production by enzyme-linked immunosorbent assay (ELISA) as described below. Primers used in this study are listed in **Table 1**.

Capsule Size Measurement

This method is referenced to previous studies with modification (28). Briefly, *C. neoformans* was incubated at 37°C in YPD broth with shaking for 24 h. Wash with PBS three times. Count the cells and resuspend at 2×10^5 cells/2 mL in Dulbecco's minimal essential media (Gibco) following incubation at 37°C and 5% CO_2 for 3 days. Cells were harvested by centrifuging. Remove all but 5–10 μL supernatant. Carefully resuspend the cells and stain with India ink on a glass microscope slide. Cover and seal. Images (>50 cells/strain) were acquired using a Zeiss light microscopy (ZEISS, AXIO) and diameters were measured using VistarImage software (x64,4.0.10289.20171114).

Intratracheal Inoculation of *C. neoformans*

Mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg body weight) plus xylazine (6.8 mg/kg) and were restrained on a foam plate. A small incision was made through the skin and the underlying salivary glands and muscles were separated to expose the trachea. Total 10^4 CFU of *C. neoformans* (3.3×10^5 yeast/ml in 30 μl) were intratracheally injected into the lungs. Culturing and trypan blue staining of the inoculated solutions were used to confirm the viability of yeast. After inoculation, the skin was closed with cyanoacrylate adhesive and the mice were monitored during recovery from the anesthesia.

In vitro Virulence Assays

To evaluate growth in liquid YPD media at 37°C, H99-α and H99 culture were serially diluted and plated on SDA for 3 days. To evaluate growth on agar plates under different conditions, the yeast H99-α and H99 cells were firstly cultured to saturation in YPD broth at 30°C, harvested and washed twice with 1×PBS buffer. The yeast cells were serially diluted and spotted on different stress media. For the oxidative and NO stress tests, the cells were incubated on Yeast Nitrogen Base (YNB) agar containing 2 mM H_2O_2 or 0.75 mM NaNO2 (pH 4.0). To evaluate the response to high salt and osmotic stresses, 1.5 M NaCl or 1.5 M sorbitol was added to the YPD agar. To analyze melanin production, *C. neoformans* strains were spotted on L-DOPA and caffeic acid medium and incubated for 5 days at 30°C (29). For capsule production, fungal cells were incubated on DMEM medium for 3 days in the presence of 5% CO_2 at 37°C and the capsule was stained with India ink. For urease assay, strains were cultured onto Christiansen's urea agar at 30°C for 24 h.

Macrophage Killing Assays

Bone marrow-derived macrophages (BMMs) were generated as previously described (30). To evaluate the fungicidal activity of BMMs to H99 and H99-α, 1×10^5 BMMs were seeded on a 96-well plate and infected with *C. neoformans* yeast (1×10^4) with or without IFN-γ (100 ng/ml). After a 24-h incubation, the total yeasts were collected by lysing BMMs with 0.5% SDS and enumerated by plating onto SDA plates. Colony values in individual groups were compared with culture wells without BMMs and expressed as a percentage of growth inhibition.

Leukocytes Isolation From the Lungs

Mice were euthanized with CO_2 and then perfused with PBS. The lungs were aseptically removed, minced with scissors, transferred to GentleMACs C tubes containing 5 ml of digestion buffer [RPMI 1640, 5% FBS, penicillin, and streptomycin; 1 mg/ml collagenase A (Roche Diagnostics); and 30 μg/ml DNase I]. After incubation at 37°C for 35 min, the cell suspension and tissue fragments were further dispersed on a GentleMACs homogenizer (Miltenyi). Erythrocytes in the cell pellets were lysed by addition of 3 ml NH4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2EDTA, pH 7.4) for 3 min. Cells were re-suspended and subjected to syringe dispersion and filtered through a sterile 100-μm nylon screen (Nitex). The filtrate was centrifuged for 30 min at 1,500 × g with no brake in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Total cell numbers were determined by counting live cells on a hemocytometer with trypan blue. Percentages of leukocyte subsets were calculated by multiplication of total cell number recovered from dispersed lungs multiplied by percentages of each subset defined via flow cytometric analysis using a set of specific markers, listed below.

For enrichment of adherent macrophages from the lungs, digested lung leukocytes were incubated for 90-min on tissue culture plastic. Non-adherent cells were rinsed away as previously described (31). Remaining macrophages were collected in Trizol reagent.

Ag-Specific Cytokine Production by Lung Leukocytes

Isolated lung leukocytes were cultured in media with heat-killed H99 in a ratio of 1:10 in 6-well plates with 2 ml

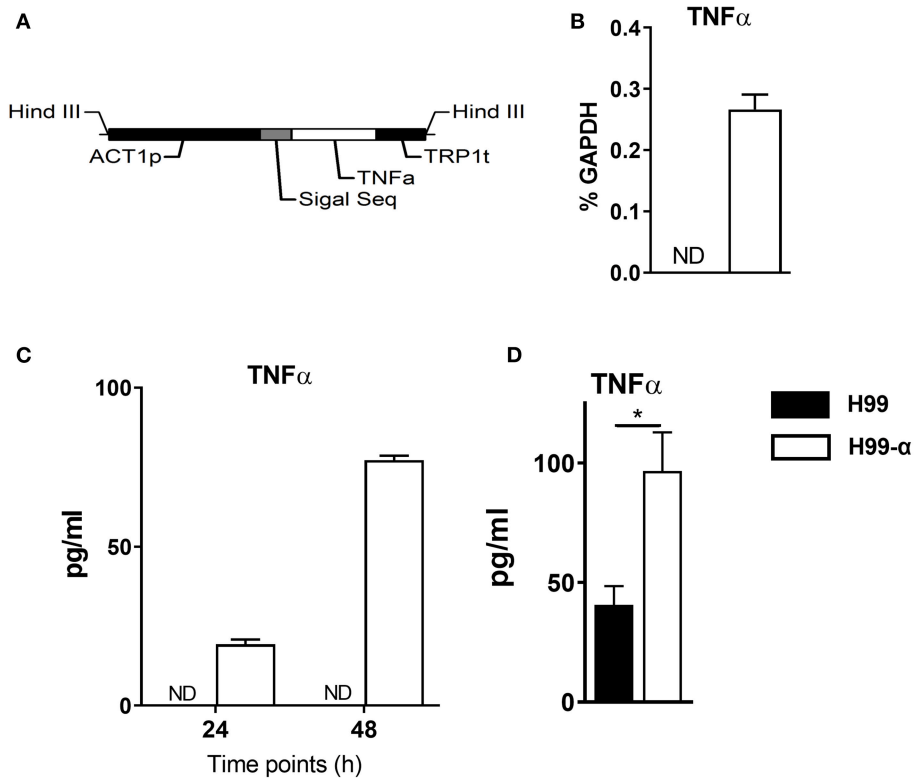

FIGURE 1 | Construction of the murine TNF-α-producing transgenic *C. neoformans* strain. **(A)** expression construct containing *C. neoformans ACTIN* promoter and murine TNF-α cDNA sequences. **(B,C)** TNF-α expression by the transgenic strain H99-α was detected by RT-qPCR or by ELISA in the culture media at 37°C. **(D)** TNF-α level in the lung homogenates of mice infected with H99-α or H99 intratracheally at 48 h post-infection (Results represent mean ± SEM; $*P < 0.05$).

complete RPMI 1640 medium at 37°C and 5% CO_2 for 48 h. Supernatants were stored and analyzed using a LEGENDplex cytometric bead array (CBA) kit (BioLegend, San Diego, CA, USA) following the manufacturer's specifications and read on an LSRII flow cytometer (Becton, Dickinson Immunocytometry Systems, Mountain View, CA, USA). The analysis was performed using BioLegend's LEGENDplex software.

RT-qPCR

Total RNA was prepared using TRIzol reagent (Invitrogen), and the first-strand cDNA was synthesized using Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Relative gene expression was quantified with SYBR green-based detection (Alkali Scientific) using a light cycler96 system (Roche) according to the manufacturer's protocols. Relative gene expression was normalized to *GAPDH* mRNA using the $2^{-\Delta Ct}$ methods. For some experiments, β-actin and 18S rRNA were also used as additional housekeeping genes to validate results calculated relative to GAPDH.

Flow Cytometric Analysis

Ab cell staining was performed as previously described (32). For staining, cells were isolated, washed in PBS, and then stained with Live Dead Fixable Aqua (Life Technologies) for 30 min. Cells were then stained with antibodies (see below), washed, and fixed in 2% formaldehyde. Data were collected on a FACS LSR2 flow cytometer using FACSDiva software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

The following gating strategy was used to identify leukocyte subsets in the lungs. First, consecutive gates identified singlets, live cells, and CD45+ leukocytes. Next, a series of selective gates were used to identify neutrophils ($CD11b^+Ly6G^+$); eosinophils ($SSC^{high}CD11c^{low}/SiglecF^+$); Alveolar macrophages ($CD11c^{high}/SiglecF^+$); $Ly6C^{high}$ monocytes ($CD11c^-/CD11b^+/Ly6C^{high}$), and DC ($CD11c^+MHCII^{high}$), Thereafter, DC were further separated into moDC, $CD11b^+$ DC and $CD103^+$ DC as follows: moDC were gated as $CD11c^+MHCII^{high}CD64^+$ cells, then remaining $CD11c^+MHCII^{high}CD64^-$ cells were further divided into $CD11b^+$ DC and $CD103^+$ DC based on the expression of SIRPα and XCR1, respectively. Isotype control antibodies were used to set gates for positive events in all flow cytometric analyses.

In vitro Macrophage Stimulation and Nitrite Production Assay

1×10^5 BMMs were seeded on a 96-well plate and infected with *C. neoformans* yeast (1×10^4). After a 24-h (or 48-h) incubation, the supernatant was centrifuged for nitrite concentration test. Briefly, 100-μL Griess reagent (0.1%

TABLE 1 | Primers used in this study.

Primer	Sequence (5′-3′)	Function
ZF0001	CAATTTCACA*GGCCATTTAGGCC*CCAATCTCGCTGACTTTCTA	Amplification of "safe haven" region
ZF0002	CATAGCTGTTTCCTGCCAATGAAGCACGCTCAAA	Amplification of "safe haven" region
ZF0003	TGGCCGTCGTTTTACCTTTGGCCAATCTCTTTCAC	Amplification of "safe haven" region
ZF0004	TCACGACGTT*GGCCATATAGGCC*TTTCCATCAGTAACATCGGG	Amplification of "safe haven" region
ZF0005	GTAAAACGACGGCCAG	Amplification of *NAT* gene
ZF0006	CAGGAAACAGCTATGAC	Amplification of *NAT* gene
ZF0007	TGATTACGCC*AAGCTT*GCTGCGAGGATGTGAGCT	Amplification of ACT1 promoter region
ZF0008	GTAGCCGTGGCGATTGACATAGACATGTTGGGCGAGT	Amplification of ACT1 promoter region
ZF0009	CTTTGGAGTCATTGCTCTGTGAGTGAAGGCGGTAAGGGGTT	Amplification of TRP1 terminator region
ZF0010	GCTCGGTACC*AAGCTT*GGTTTATCTGTATTAACACGGA	Amplification of TRP1 terminator region
ZF0011	AC*GGCCTATATGGCC*AACGTCGTGACTGGGAA	Constructing of plasmid (pCH234)
ZF0012	TGGGCCTAAATGGCCTGTGAAATTGTTATCCGCTC	Constructing of plasmid (pCH234)
GAPDH-F	TATGTCGTGGAGTCTACTGGT	qPCR
GAPDH-R	GAGTTGTCATATTTCTCGTGG	qPCR
ARG1-F	CAGAAGAATGGAAGAGTCAG	qPCR
ARG1-R	CAGATATGCAGGGAGTCACC	qPCR
iNOS-F	GCATTGGAAGTGAAGCGTTTC	qPCR
iNOS-R	GGCAGCCTGTGAGACCTTTG	qPCR

naphthyl ethylenediamine in water and 1% sulfanilamide in 5% orthophosphoric acid; Sigma, Aldrich, St. Louis, MO) was added to 100-μL experimental supernatants or 100-μL sodium nitrite standard (0–100 μmol/L). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 550 nm. Nitrite concentrations of experimental samples were determined by reference to a standard curve constructed in parallel. BMM cells were lysed using TRIzol reagent (Invitrogen), following RNA extraction and RT-PCR as described above.

Calculations and Statistics

Values are reported as the arithmetic mean ± standard error of the mean. Student *t*-test or two-way ANOVA with a Bonferroni *post-hoc* test were used for comparisons of individual means. All experiments were repeated at least three times. Statistical calculations were performed using GraphPad Prism version 6.00. Means with p values <0.05 were considered significantly different.

RESULTS

Transgenic Construct *C. neoformans* H99-α Produces Murine TNF-α

We generated an expression construct in which *C. neoformans ACTIN* promoter was used to drive the expression of the murine TNF-α cDNA sequence. The signal sequence of cryptococcal *PLB1* gene was also included in the expression construct to ensure extracellular secretion of TNF-α (**Figure 1A**). The expression construct was transformed into the WT *C. neoformans* strain H99, and single insertion of murine TNFα sequences into the genome of transformant cells was confirmed by PCRs and Southern blot. One of the successfully transformed strains, designated H99-α, was selected for further study. While no TNF-α expression could be detected in the WT H99 strain, H99-α showed detectable expression of TNF-α at both transcription and protein level when cultured in liquid SDB media at 37°C (**Figures 1B,C**) To determine *in vivo* TNF-α production during infection, mice were intratracheally infected with H99-α and H99, respectively. Mice infected with H99-α showed significantly greater TNF-α production in the lung homogenates at 48 h post-inoculation compared to H99-infected mice (**Figure 1D**).

Expression of the TNF-α Has No Effect on Fungal Fitness and Expression of Classical Virulence Factors by *C. neoformans in vitro*

We next evaluated whether the expression of TNF-α affected cryptococcal fitness and virulence factor expression *in vitro*. H99-α strain showed no significant differences in growth rate in both liquid culture media at 37°C (**Figure 2A**) or on agar plates at 30°C or 37°C (**Figures 2B,C**) compared to WT H99 strain, demonstrating intact thermotolerance. H99-α also exhibited similar sensitivity to oxidative, hyperosmotic, and high salt stresses to that of the WT H99 strain (**Figures 2D–G**). Melanin (**Figure 2H**), urease (**Figure 2I**), and capsule production (**Figure 2J** and **Figure S1**) were also observed to be the same in H99-α as in the WT H99. Thus, the H99-α strain had no defects in the *in vitro* phenotypes commonly associated with cryptococcal fitness and expression of virulence factors.

Mice Showed Improved Fungal Control and Extended Survival Time During Pulmonary Infection With H99-α

Having determined that H99-α, apart from the production of murine TNFα, showed all the *in vitro*-features of the WT H99 strain, we next evaluated how TNF-α secreted by the transgenic

strain affected host responses during *C. neoformans* infection. BALB/c mice were inoculated with a dose of 10^4 CFU of either H99-α or H99 intratracheally and fungal burdens were evaluated at different time points in the lungs and brains. Mice infected with H99-α exhibited a substantial decrease (100-fold) in fungal burden in the lungs, starting from 2 days post infection (dpi) and continuing through at least 3-weeks (**Figure 3A**). Fungal CNS dissemination was also ablated in H99-α infected mice at

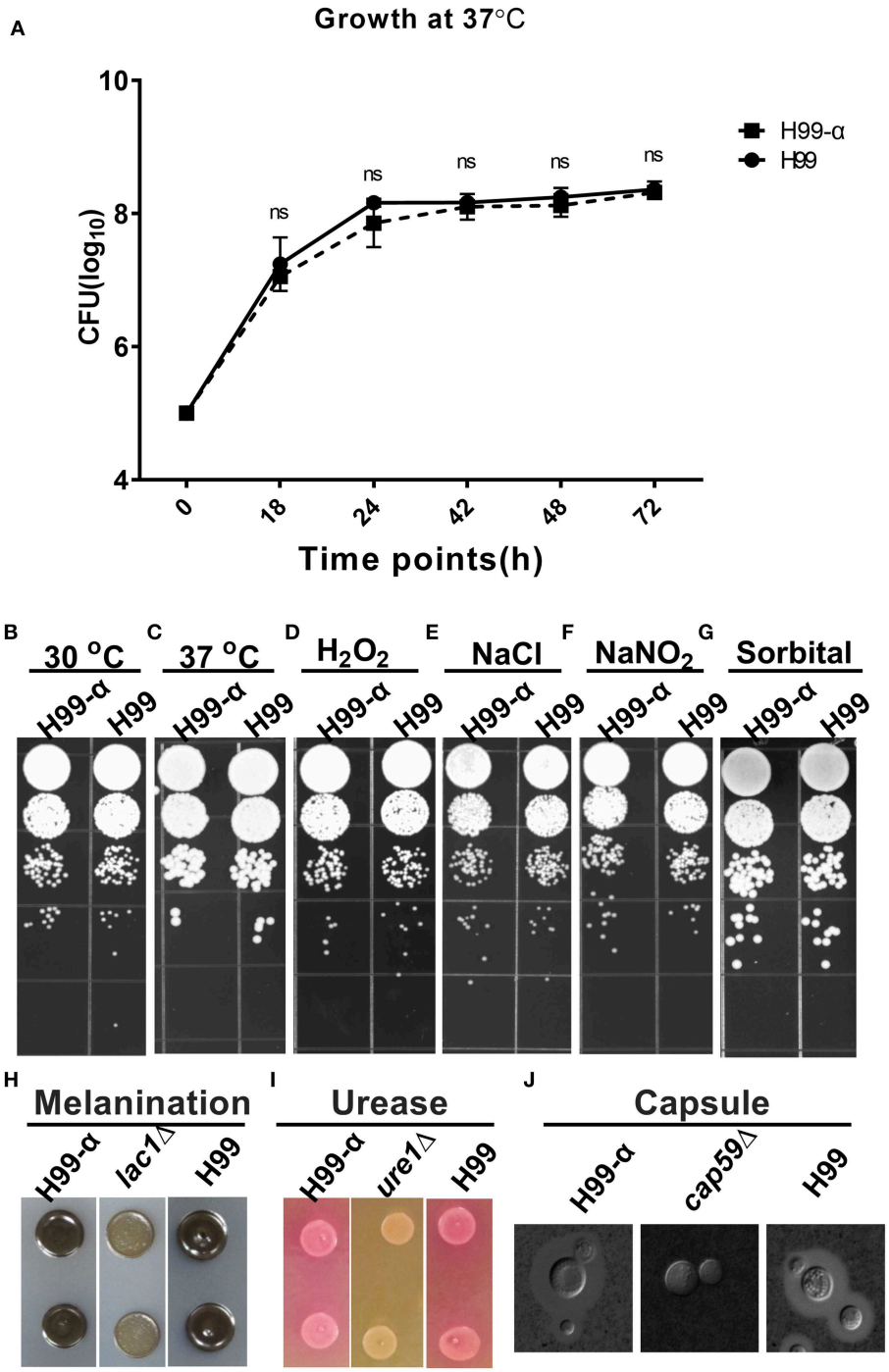

FIGURE 2 | Expression of the murine TNF-α has no effect on cryptococcal fitness and major virulence factor production *in vitro*. **(A)** Growth curve of H99-α and H99 in liquid SDB media at 37°C. **(B)** Growth of H99-α and H99 on SD agar at 30°C **(B)** and 37°C **(C)**, and agar plates containing H_2O_2 **(D)**, NaCl **(E)**, $NaNO_2$ **(F)**, sorbitol **(G)**. H99 and H99-α melanin production **(H)**, urease activity **(I)**, and capsule formation **(J)** with negative controls *lac1*Δ, *ure1*Δ, and *cap59*Δ for **(H–J)**, respectively. Note that TNF-α expression by H99-α has not affected cryptococcal fitness or the expression of major virulence factor (Results represent mean ± SEM; ns, no Significance).

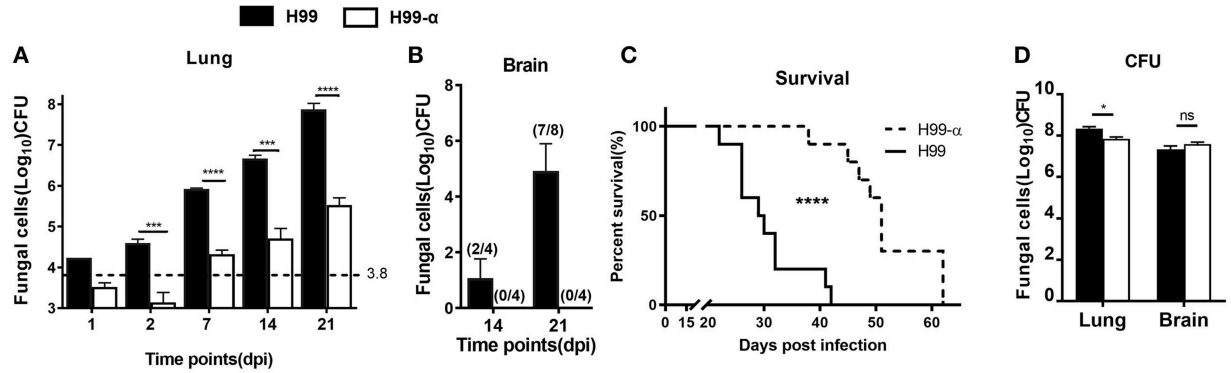

FIGURE 3 | Induction of TNF-α by the transgenic strain improve survival and fungal containment in comparison with the WT strain. BALB/c mice were infected with an intratracheal inoculation of 10^4 cells of H99-α or WT *C. neoformans* strain H99. Fungal burden from lungs **(A)** and brains **(B)** were quantified at different time points. **(C)** Mice survival was evaluated. $N = 10$ mice per group. **(D)** H99-α infected mice showed comparable fungal burden in both lungs and brains with H99 at time of death. BALB/c mice were infected with an intratracheal inoculation of 104 cells of H99-α or WT *C. neoformans* strain H99. Fungal burden from lungs and brains were quantified when mice dead (weight loss ≥ 20%). Note that the number on the graph **(A)** indicates in the initial inoculation dose of fungal organisms. The numbers on the graph **(B)** indicate the number of mice which are detected with fungal dissemination out of the group size (Results represent mean ± SEM; $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

14 and 21 dpi (**Figure 3B**). Furthermore, H99-α infected mice showed a much longer survival time with a median survival time of 51 days, compared with 27 days in H99 infected group (**Figure 3C**). We further evaluated the fungal burden in the lungs and brains of mice from both groups when mice succumbed to infection. We found that both groups of mice showed overwhelmingly high lung fungal burdens and a profound level of CNS dissemination at the time of death (**Figure 3D**). While pulmonary fungal load in H99-α-infected mice was slightly but significantly lower compared to the H99-infected group, there was no difference CNS burden between groups at mice harvested at the end-stage of the disease (**Figure 3D**). Taken together, our results suggested that murine TNF-α production by highly virulent of *C. neoformans* H99 can enhance host anti-cryptococcal defenses and fungal containment, but ultimately cannot oppose uncontrolled expansion and dissemination of cryptococcal organism in the infected host.

Fungal Containment of TNF-α Producing Transgenic Strain Limits Lung Pathology in the Infected Lungs

TNF-α is a potent pro-inflammatory mediator and the development of excessive inflammation could be a crucial concern while using TNF-α-based immunotherapy. Thus, our next goal was to determine the effect of TNF-α-producing strain on the extent of the inflammatory response and pathology in the lungs of infected mice. Our results supported that TNF-α induction by *C. neoformans* have not produced these undesirable effects. The lungs from H99 infected-mice showed a high level of inflammatory infiltrates at 14 dpi, however, most of the organisms within the alveolar space were not contained by the immune cells (**Figures 4A,C**). The cellular infiltrates contained predominantly cells with myeloid morphology (granulocytes and macrophages), consistent with previous reports. In contrast, H99-α infected mice showed nice compartmentalization of the infected and uninfected areas. Most parts of the lung tissues remained healthy and "clean" in comparison with the H99-infected mice. The fungal organisms were all contained within a confined area surrounded by dense inflammatory infiltrates, mostly lymphocytes and mononuclear cells (**Figures 4B,D**), consistent with improved host defenses in H99-α-infected group. No evidence of uncontrolled inflammation or inflammatory lung damage has been noted for up to 3 weeks of infection. These results demonstrate that the transgenic strain H99-α induces more focal and effective response compared to strain H99, which limits lung pathology at the corresponding time points.

H99-α-Infected Mice Showed Selectively Increased Frequencies of T Cells and the Decreased Frequency of Eosinophils in the Infected Lungs Compared to H99-Infected Mice

The histology results demonstrated that inflammatory recruitment pattern in the lungs infected with H99 and H99-α were different. To quantify cellular responses in the lungs infected with H99-α and H99, we isolated leukocytes from the dispersed lungs and conducted flow cytometry analysis. We found a reduced accumulation of total lung leukocyte numbers at 14 and 21 dpi in H99-α-infected mice (**Figure 5A**), which is consistent with our histology study. H99-α-infected mice showed the lower absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells, B cells, alveolar macrophages, and monocytes compared to H99-infected mice, but more profound reductions were found in quantities of eosinophils and neutrophils (**Figures 5B–H**). We further found much higher frequencies of lymphoid cells of the adaptive immune system ($CD4^+$ T cells, $CD8^+$ T cells and B cells) in H99-α infected lungs compared to H99-infected lungs

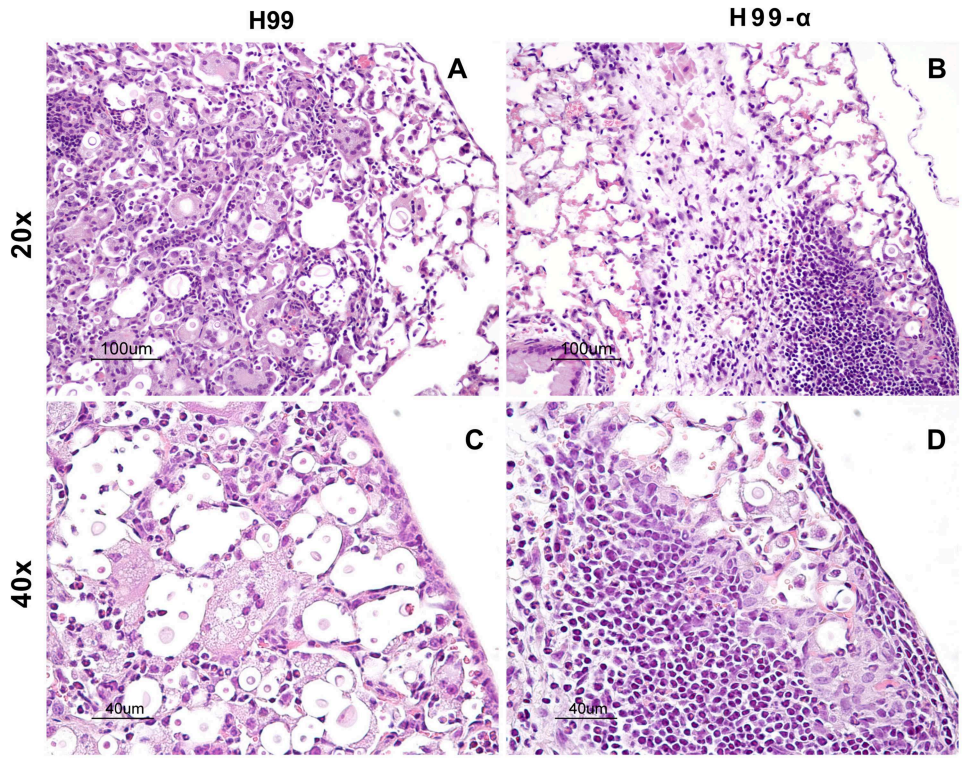

FIGURE 4 | Production of TNF-α by the transgenic strain does not induce excessive inflammatory response or severe pathology in the infected lungs. Lungs from mice infected with H99-α or H99 were harvested at 14 dpi followed by Haemotoxylin & Eosin staining. The photographs were taken at ×20 **(A,B)** and ×40 objective power **(C,D)**. Note that H99-α-infected lungs showed different inflammatory features such as improved containment of the yeast, more condensed leukocytes infiltration around the infection site and less pronounced granulocyte responses than H99-infected lungs.

(**Figures 5I,J**). In contrast, H99 responses were dominated by myeloid cell populations, especially granulocytes (eosinophils and neutrophils) (**Figures 5I,J**). Collectively, these data show that H99-α skews the inflammatory response from myeloid cell-dominant toward lymphocyte-dominant pattern with a significant reduction in eosinophil accumulation.

TNF-α Production by Transgenic *C. neoformans* Strain Shifts Cytokine Balance Away From Non-protective Type 2 Bias During Cryptococcal Infection

The changes in the cellular composition of the inflammatory cell infiltrate (notably, the reduced eosinophil accumulation) during H99-α infection suggested that production of TNF-α by the transgenic strain modulated Th1/Th2 bias and Th1/Th2-associated cytokine production in the lungs. We further analyzed the cryptococcal antigen-induced cytokine production by leukocytes from lungs at 14 dpi. Dispersed pulmonary leukocytes were adjusted to the same concentration, pulsed with the equivalent amount of heat-killed H99 and cultured for 24 h. Interestingly, lung leukocytes from H99-α and H99-infected mice showed a similar level of Th1 cytokines expression, including IFN-γ and TNF-α (**Figures 6A,B**), whereas expression of Th2 cytokines was profoundly diminished in H99-α-infected group compared with H99-infected group (**Figures 6C–E**). To assess if murine TNF-α expression by H99-α altered the overall Th1/Th2 balance, we evaluated the ratio of Th1/Th2-type cytokines production. As shown in **Figure 6F**, H99-α-infected mice significantly skewed the Th cytokine balance in a way that suggests more protective Th1 polarization, indicating a protective role of the TNF-α-producing transgenic *C. neoformans* strain in the efferent phase of infection. Finally, we performed qPCR analysis on adherence enriched macrophages from the infected lungs of H99-α and H99 infected mice (**Figure S2**). Interestingly, no significant difference was detected in IFN-γ or TNF-α expression; however, we found that the expression of IL-13 was significantly decreased in H99-α infected group compared to H99 infected group. Thus, the production of murine TNF-α by *C. neoformans* promoted protective altered cytokine profile shifting the balance form non-protective Th2 cytokine profile toward more protective.

Production of TNF-α by the H99-α Transgenic Strain Enhances Activation of cDC1 and Ly6C$^+$ Monocyte at the Efferent Phase of Cryptococcal Infection

Having found that the H99-α prevented non-protective pulmonary type-2 cytokine bias, we asked whether this

favorable shift in pulmonary immune responses was related to improved activation of lung antigen presenting cells. We analyzed monocyte and DC subsets: $CD103^+$ cDC1, $CD11b^+$ cDC2, as well as monocyte-derived DC (moDC) activation using CD80 and MHC class II molecule surface expression as readouts. H99-α infected mice showed elevated expression of CD80 in $CD103^+$ cDC1 compared with H99 infected group at both 14 and 21 dpi (**Figures 7A,B**) and increased expression of MHC class II in monocytes subsets at 21 dpi compared with WT strain (**Figures 7C,D**). However, we did not observe any difference in activation in either CD11b+ cDC2 or moDC between H99-α and H99 infected mice (**Figure S3**). Collectively, our data demonstrate that TNF-α induced by H99-α may specifically enhance co-stimulatory activation of cDC1 and MHCII in monocyte during pulmonary cryptococcosis.

Expression of TNF-α by the H99-α Transgenic Strain Diminishes Alternative Activation of Lung Macrophages and Promotes the Anti-cryptococcal Activity of Bone Marrow-Derived Macrophages in the Presence of IFN-γ

Cytokine bias in the infected lungs directs macrophage activation patterns: classical activation (M1) leads to fugal containment and clearance, while alternative activation (M2) is non-protective and linked to progressive cryptococcosis (2). We thus examined

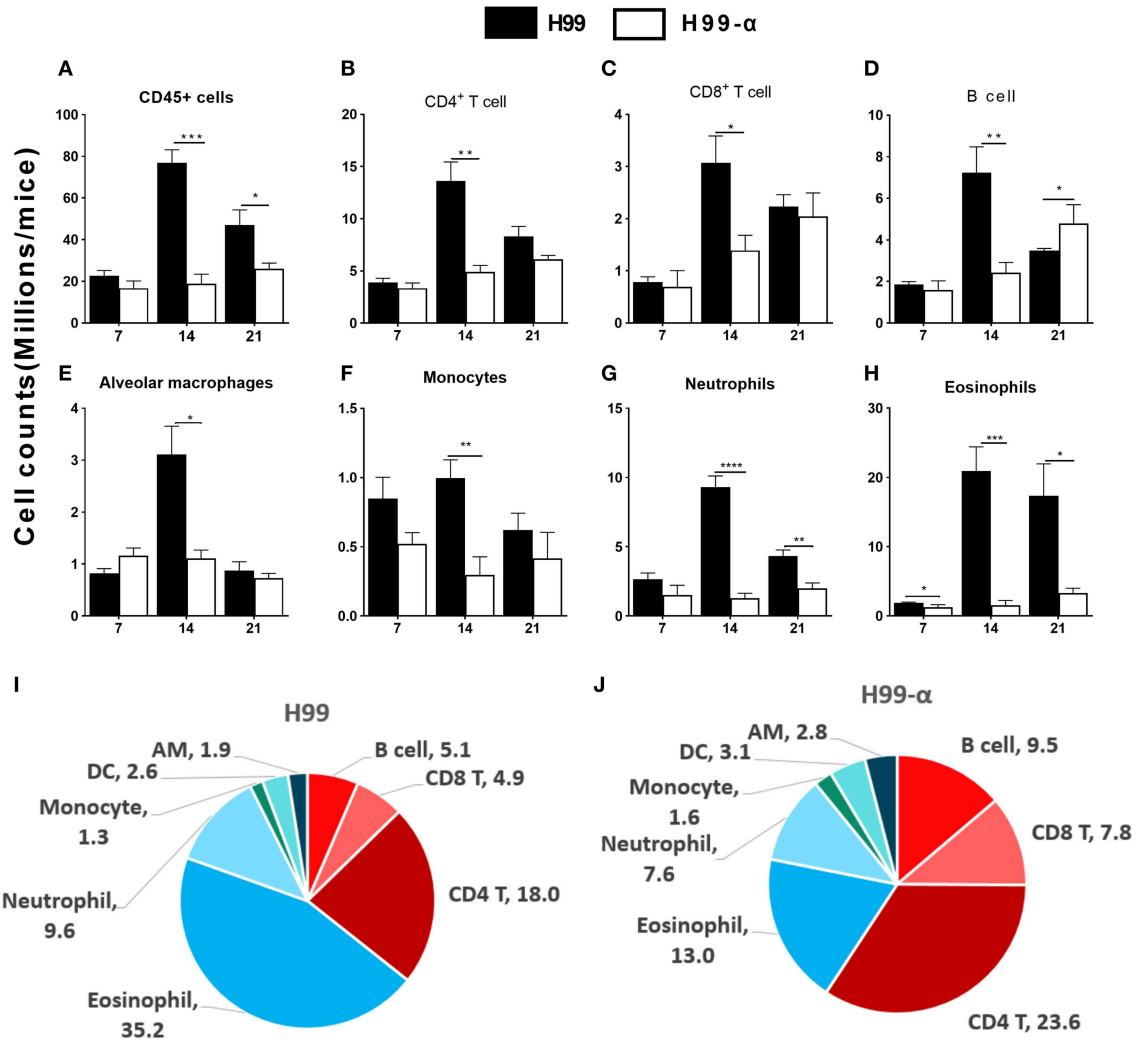

FIGURE 5 | Production of TNF-α by the transgenic strain demonstrated a higher frequency of lymphocyte population than WT H99 stain. The subsets of immune cells in the infected lungs were analyzed by flow cytometry. $CD45^+$ cell counts were calculated as total inflammatory infiltrates in the lungs after infection **(A)**. Cell number of $CD4^+$ T cell **(B)**, $CD8^+$ T cell **(C)**, B cell numbers **(D)**, alveolar macrophages **(E)**, monocytes **(F)**, neutrophils **(G)**, and eosinophils **(H)** were compared between H99-α and H99 infected mice. Frequencies (% of the $CD45^+$ cells) of myeloid and lymphoid cell subsets in H99 **(I)** and H99-α **(J)** infected lungs at 21 dpi. Note that H99-α infected mice showed decreased leukocyte number but enhanced overall T cell and B cell frequencies in comparison with H99 group (Results represent mean ± SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ compared with H99 infected group at the same time point).

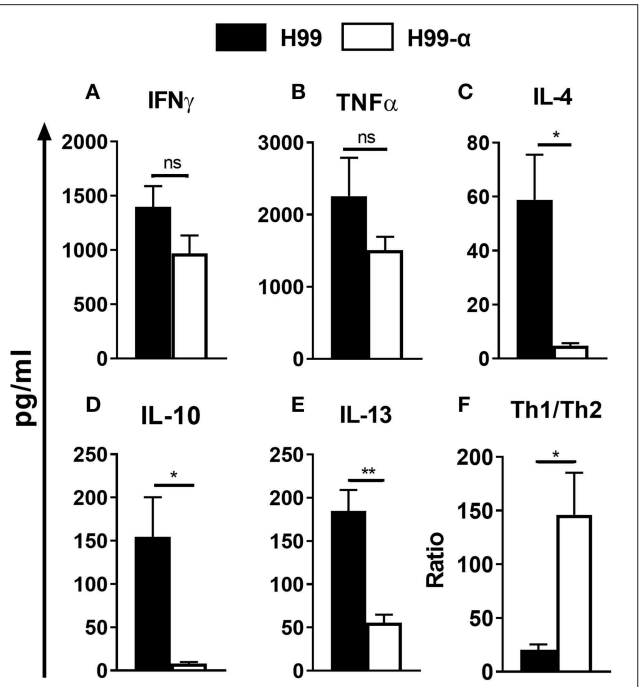

FIGURE 6 | The TNF-α-producing transgenic strain opposed the development of pulmonary Th2 cytokine bias during cryptococcal infection. Total lung leukocytes were isolated on 14 dpi and incubated with heat-killed H99. The supernatant was collected followed by CBA assay. No significant difference was detected in IFN-γ **(A)**, and TNF-α **(B)** production. IL-4 **(C)**, IL-10 **(D)**, and IL-13 **(E)** secretion were profoundly attenuated in leukocytes from H99-α infected mice compared to WT H99-infected mice. **(F)** Th1/Th2 bias ratio was calculated as (IFN-γ+TNF-α)/(IL-4+IL-10+IL-13). Results represent mean ± SEM, $N = 4$ or more mice per group (*$P < 0.05$; **$P < 0.01$; ns, no significant difference).

the transcriptional expression of M1 hallmark gene nitric oxide synthase (iNOS) and M2 hallmark gene Arginase (Arg1) by adherence-enriched macrophages in the H99-α and H99 infected lungs. Arg1/iNOS ratio was also calculated as a readout of M2 vs. M1 bias. Macrophages from the H99-α infected lungs showed significantly lower expression of both *iNOS* and *ARG1* (**Figures 8A,B**), suggesting that both classical (M1) and alternative (M2) activation genes were upregulated to a lesser degree. This was consistent with significantly lower antigen load and less vigorous lung inflammatory response in the H99-α-infected group compared with H99-infected group. However, the Arg1/iNOS ratio was dramatically decreased in the H99-α-infected group relative to the control group (**Figure 8C**), suggesting that H99-α shifted overall macrophages polarization balance toward a less pronounced M2 activation.

While the observed changes in the adaptive immune response typically require time for the development of the immune response, our study showed significantly improved containment of H99a as early as 2 dpi. To determine if murine TNFα production directly affected macrophage fungicidal function, we examined *C. neoformans,* and bone marrow-derived macrophage (BMMs) interactions *in vitro*. Fungal growth inhibition and fungicidal nitric oxide (NO) production by macrophages challenged with H99-α or H99 were compared. Inhibition rates of fungal growth and iNOS mRNA expression were similar between macrophages challenged by H99-α or H99 in the absence of cytokine stimulation (**Figure 8D**). BMMs incubated with H99 or H99-α showed similar TNF-α mRNA expression (**Figure S4**). However, in the presence of IFN-γ, we observed significantly greater inhibition of *C. neoformans* by macrophages challenged with H99-α compared to macrophages challenged with H99. Consistently, we detected enhanced iNOS mRNA expression and nitric oxide production in macrophages challenged with H99-α compared to those challenged with H99 in the presence of IFN-γ stimulation (**Figures 8E,F**). While Arg1 expression level was similar between groups, the Arg1/iNOS ratio was significantly diminished in the H99-α infected group relative to the control group in the presence of IFN-γ stimulation (**Figures 8G,H**). The supplementation with exogenous TNF-α in addition to IFN-γ (TNF-α + IFN-γ BMM groups) resulted in most enhanced killing capacity and iNOS production by BMM that was no longer different in cells infected with H99 and H99-α (**Figures 8D–H**). These results demonstrate that TNF-α produced by H99-α, on its own, cannot improve the fungicidal function of macrophages, but it enhances these effects in the presence of IFN-γ activation to the similar degree as the exogenously added TNF-α.

DISCUSSION

The goal of this study was to enhance understanding of the role of TNF-α in anti-cryptococcal host defenses and to evaluate its supplementation as a strategy to enhance immunization against *C. neoformans.* To this end, we have engineered a *C. neoformans* H99-α strain capable of expressing murine TNF-α and explored its efficacy to stimulate protective anti-cryptococcal host responses. We demonstrate that mice infected with *C. neoformans* H99-α have vastly improved fungal containment in the infected lungs, showed limited lung pathology, resisted CNS dissemination, and gained a substantial survival benefit. These improved clinical outcomes were mechanistically linked to (1) the altered inflammatory responses in the lungs, with a selective increase in proportions of the adaptive immune cells (T cells and B-cells) and diminished non-protective characteristics such as eosinophilia; (2) significantly diminished non-protective Th2-type cytokine expression and thereby improved Th1/Th2 cytokine ratio; (3) and improved balance of M1/M2 macrophage polarization *in vivo* coupled with the improved *in vitro* fungicidal activity of primary macrophages against H99-α relative to H99, in the presence of IFN-γ stimulation.

Our studies found that the transgenic strain with TNF-α production enhances fungal containment by the host at the very early innate phase of infection. The growth of H99-α compared to H99 was significantly suppressed at 2 dpi and showed a strong trend for reduced fungal burden at day 1. This improved control of the transgenic strain is unlikely to result from attenuation of the strain itself following the integration of the TNF-α expression construct. We have found that the transgenic stain showed similar viability and growth rate at both

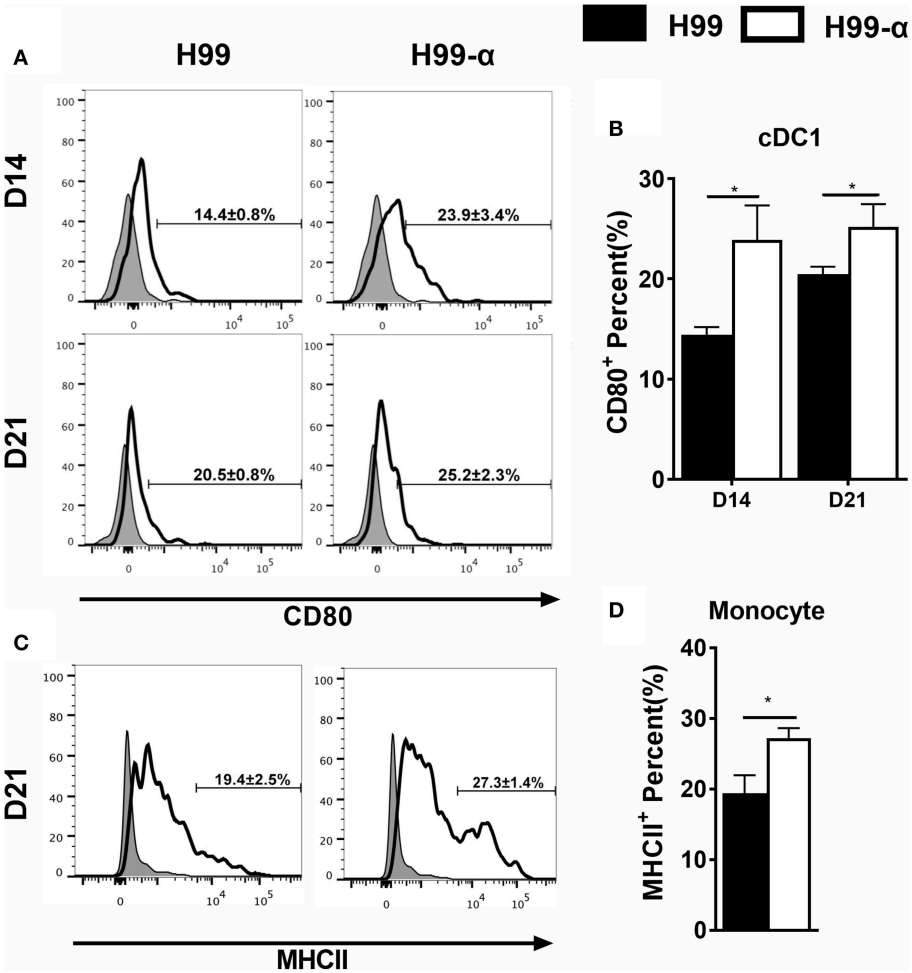

FIGURE 7 | H99-α infected mice showed higher cDC1 and monocyte activation compared with H99 infected mice. Isolated lung leukocytes from infected mice were analyzed by flow cytometry. The surface expression levels of CD80 or MHCII were measured. **(A,B)** H99-α induced a higher frequency of cDC1 population producing CD80 on 14 and 21 dpi. **(C,D)** H99-α induced elevated frequency of monocytes producing MHCII at 21 dpi. Results represent mean ± SEM; $N = 4$ or more mice per group (*$P < 0.05$).

30°C and 37°C, unaltered expression of urease, laccase (melanin) and capsule, and similar sensitivity to the oxidative, high salt and osmotic stresses compared to the WT strain H99. Instead, this rapid suppression of fungal CFU strongly suggests that TNF-α production by H99-α enhanced the fungicidal function of the resident cells (macrophages and DC) since these effects became apparent even prior to the expected wave of leukocyte recruitment. The *in vitro* results also support that the effect of fungal-derived TNF-α on the fungicidal function of macrophages can be detected as early as 24 h, provided that an additional activating signal such as IFN-γ is present. Overall, these findings support that TNF-α expression by H99-α effectively activates the innate defenses to substantially reduce fungal burden right at the onset of the infection.

While the effects of TNF-α at the early defenses were insufficient to eliminate the infection with H99-α during the innate-phase, additional benefits of TNF-α production were observed during the adaptive phase of infection. Cell-mediated immunity, especially the involvement of Th1-type CD4 T cell, is the most effective host defense mechanism against *C. neoformans* infection in the lungs (2). Robust early TNF-α induction during *C. neoformans* infection stabilizes Th1 and prevent the development of non-protective Th2 (16, 17, 33). Consistently, we found that mice infected with TNF-α-producing strain showed remarkable alleviation in fungal expansion and the absence of Th2-driven lung tissue pathology in opposition to uncontrolled growth and rapid CNS dissemination of the WT H99 during the adaptive phase of host response, which is typically developed by 14 dpi (2, 13). In addition to the overall weaker inflammatory infiltration (most likely associated with much lower antigen load), H99-α shifted the immune response balance toward Th1, which is evidenced by (1) condensed immune cells infiltration around infection sites with restrained fungal dissemination; (2) altered inflammatory infiltration

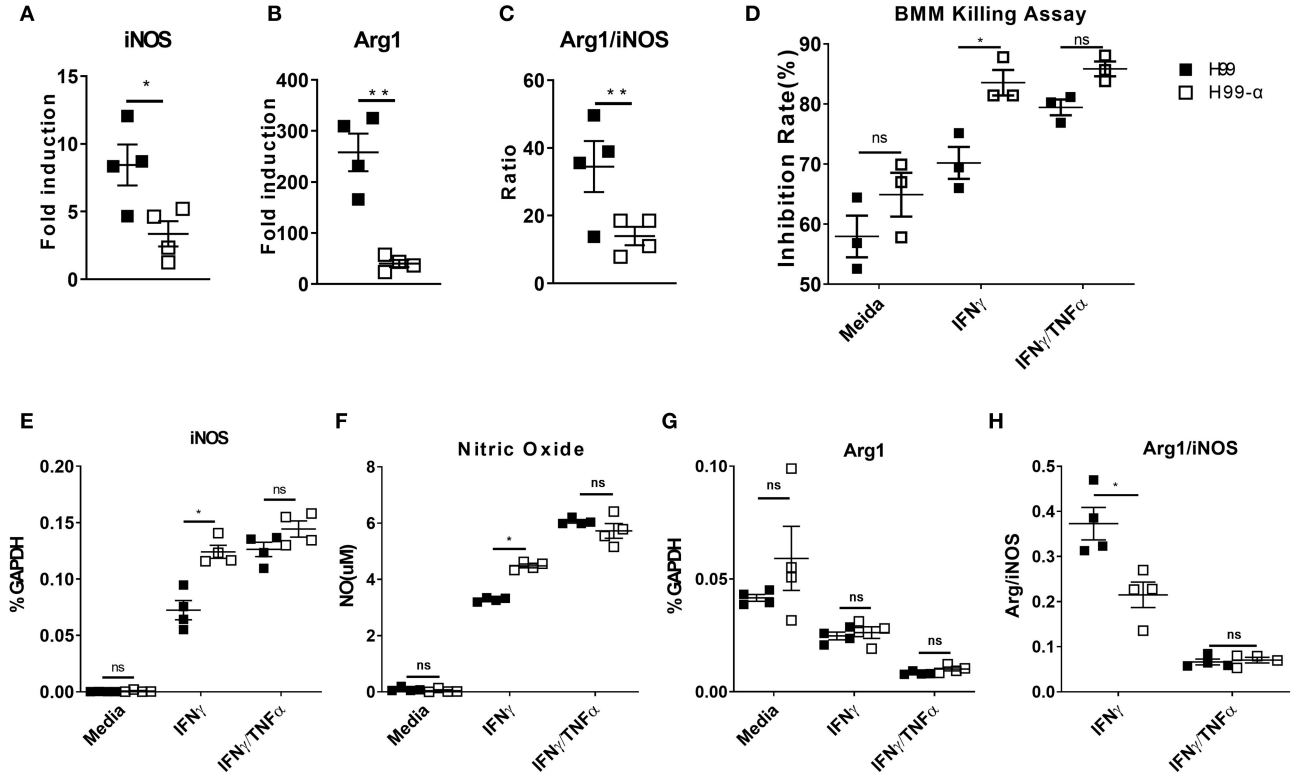

FIGURE 8 | Expression of the murine TNF-α diminishes alternative activation of lung macrophages and promotes the anti-cryptococcal activity of BMMs *in vitro*. **(A,B)** mRNA expression of iNOS and Arginase (Arg1) from adherence-enriched macrophages at 14 dpi were determined by RT-qPCR. **(C)** Arg1/iNOS ratio was calculated as a readout of M2 vs. M1 activation bias. BMMs were co-cultured with mouse serum (10%) opsonized H99α or H99 in the medium only, IFN-γ, or IFN-γ together with TNF-α. Inhibition rates of fungal growth **(D)**, iNOS mRNA expression by BMM **(E)**, NO production in the supernatant **(F)**, Arg1 mRNA expression **(G)**, and Arg1/iNOS ratio **(H)** were evaluated. Notably, significantly higher growth inhibition rate, iNOS expression and NO production were found in macrophages challenged with H99-α than H99 in the presence of IFN-γ. Results represent mean ± SEM, $N = 4$ for each group (*$P < 0.05$; **$P < 0.01$; ns, no significant difference).

phenotype from eosinophil and myeloid cell-dominant toward lymphocyte-dominant pattern; and (3) the improved Th1/Th2 cytokine ratio.

The significant role of TNF-α in the generation of protective Th1 response in cryptococcal pneumonia have been demonstrated in several models. Most recently, we have reported that early TNF-α signaling plays a critical role in classical DC activation and subsequent Th1/Th17 immune responses in CBA/J mice infected with a moderately virulent strain *C. neoformans* 52D (34). Furthermore, delivery of a TNF-α-expressing adenoviral vector to C57BL/6 mice infected with *C. neoformans* 52D prevented Th2 development and promoted protective Th1 responses during cryptococcal infection, which was associated with improved pulmonary DC MHCII-maturation (35). In the present study, we have used a highly virulent strain H99, which induces strong Th2 bias as a part of its virulence strategy (11, 13, 36, 37). In keeping with previous findings, direct TNF-α production by yeast strain modulated Th1/Th2 balance during infection, and drove the improved co-stimulatory activation of CD103+ cDC1, reported to be specialized in promoting Th1 response (38). However, the predominant effect of TNF-α production was significant ablation of the Th2-arm without a concurrent amplification of the pulmonary Th1 cytokine expression. Thus, despite the significant improvement in overall Th1/Th2 cytokine balance, the H99-α infected mice were unable to develop a robust protective response and resolve the infection. This is further supported by high lung and brain fungal burdens detected in H99-α infected mice comparable to WT infected mice at the time of death. Altogether, our data overwhelmingly suggests that the mortality of H99-α infected mice was a result of insufficient host's control of the fungal growth that occurred despite somewhat improved immune response. Thus, cryptococcal expression of TNF-α is unlikely to become an optimal tool to induce complete immunoprotection against *C. neoformans* infection, in contrast with findings obtained with IFN-γ-producing *C. neoformans* strain, H99-γ (24). Primary infection with H99-γ not only becomes completely resolved but it also confers protection against a secondary challenge with the WT H99 strain (24). This comparison indicates that TNF-α ranks below IFN-γ when it comes to its importance as a protective cytokine. In this respect, the importance of TNF-α appears to match that of IL-12, of which supplementation results in improved survival and fungal

control but not a complete clearance (39). At the cellular level, this is illustrated by the requirement of IFN-γ to unmask the beneficial effect of TNF-α for macrophage fungicidal activity *in vitro*.

Together, our studies show that TNF-α itself produced by transgenic *C. neoformans* strain is not sufficient to confer complete protection against highly virulent strain H99, despite the fact that early TNF-α production is critical for the development of protective responses during cryptococcal infection (16). Although in the current study we have not addressed several limitations, such as TNF-α delivery in clinical settings, the effects of increased dose of TNF-α supplementation, as well as the potential side effects of TNF-α that could come with higher doses of H99-α strain, our study opens a possibility that H99-α could become a beneficial "enhancer" when used in combination with H99-γ for vaccination strategy. Future studies are needed to determine whether combined immunization with strains H99-α and H99-γ would result in further improvement of protective effects induced by H99-γ alone.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All experiments were approved by the Veterans Affairs Institutional Animal Care and Use Committee under protocol number 0512-025 and were performed in accordance with NIH guidelines and the Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

MO, WL, and WF designed and supervised the project. ZF, JX, and JY performed the experiments, collected, and analyzed data. JS and QX designed the vector. WP and RY contributed to data analysis. ZF, JX, and MO wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01725/full#supplementary-material

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ORIGINAL RESEARCH
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Reciprocal Inhibition of Adiponectin and Innate Lung Immune Responses to Chitin and *Aspergillus fumigatus*

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Chitin is a structural biopolymer found in numerous organisms, including pathogenic fungi, and recognized as an immune-stimulating pathogen associated molecular pattern by pattern recognition molecules of the host immune system. However, programming and regulation of lung innate immunity to chitin inhalation in the context of inhalation of fungal pathogens such as *Aspergillus fumigatus* is complex and our understanding incomplete. Here we report that the systemic metabolism-regulating cytokine adiponectin is decreased in the lungs and serum of mice after chitin inhalation, with a concomitant decrease in surface expression of the adiponectin receptor AdipoR1 on lung leukocytes. Constitutive lung expression of acidic mammalian chitinase resulted in decreased inflammatory cytokine gene expression and neutrophil recruitment, but did not significantly affect lung adiponectin transcription. Exogenous recombinant adiponectin specifically dampened airway chitin-mediated eosinophil recruitment, while adiponectin deficiency resulted in increased airway eosinophils. The presence of adiponectin also resulted in decreased CCL11-mediated migration of bone marrow-derived eosinophils. In contrast to purified chitin, aspiration of viable conidia from the high chitin-expressing *A. fumigatus* isolate Af5517 resulted in increased neutrophil recruitment and inflammatory cytokine gene expression in adiponectin-deficient mice, while no significant changes were observed in response to the isolate Af293. Our results identify a novel role for the adiponectin pathway in inhibition of lung inflammatory responses to chitin and *A. fumigatus* inhalation.

Keywords: adiponectin, chitin, *Aspergillus fumigatus*, lung immune responses, inflammatory cytokines, eosinophils, neutrophils

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INTRODUCTION

Chitin is a ubiquitous biopolymer of high-tensile strength found in many eukaryotes, including fungi, crustaceans, arthropods, and helminths (1–3). In contrast, chitin synthesis is absent in mammalian tissues, where chitin-degrading chitinases are widely expressed and highly conserved (4). Defects in chitinase-mediated chitin degradation are associated with airway accumulation of chitin, inflammatory cells, and development of pulmonary fibrosis (5). Pattern recognition of chitin particles by an array of other microbial ligand-binding receptors drives immune responses that vary based on chitin particle size and acetylation, ranging from inflammatory to regulatory profiles

(2, 6, 7). In addition, inflammatory cytokine expression in response to purified or fungal chitin exposure includes secretion of IL-1, IL-4, IL-13, IL-10, and IL-17 (6, 8, 9). However, programming and regulation of lung innate immunity to chitin inhalation in the context of inhalation of fungal pathogens such as *Aspergillus fumigatus* is complex and our understanding incomplete.

The adipose tissue cytokine adiponectin is well-known as a regulator of insulin responsiveness and fatty acid oxidation, yet also exerts anti-inflammatory effects on macrophages, innate lymphocytes, eosinophils, and neutrophils (10). Moreover, adiponectin is constitutively high in the plasma of healthy, lean individuals (11–13), and significantly decreased in association with the chronic inflammatory diseases obesity and asthma (14–16). Adiponectin deficiency resulted in increased lung inflammation and pathology in mouse models of allergic asthma and ozone inhalation, supporting an anti-inflammatory role for adiponectin in lung immune pathology (17, 18). Recently, obese and adiponectin-deficient mice were reported deficient in clearance of the food-borne bacterial pathogen *Listeria monocytogenes* due to increased bone marrow inflammation and defective granulopoiesis (19). Adiponectin inhibits macrophage activation and cytokine secretion in response to bacterial lipopolysaccharide, potentially via blockade or desensitization of inflammatory signaling pathways (10). However, the effect of adiponectin on lung inflammation induced by fungal pattern associated molecular patterns (PAMPs) is unknown.

Herein we report that lung and serum adiponectin were decreased after aspiration of purified chitin or conidia of a high chitin-expressing strain of *A. fumigatus*. Co-aspiration of chitin with recombinant adiponectin or adiponectin deficiency resulted in decreased or increased lung eosinophil recruitment, respectively, while numbers of neutrophils and alveolar macrophages were not affected. Eosinophils, neutrophils, and alveolar macrophages expressed surface AdipoR1 that was decreased upon chitin inhalation, and eosinophil migration in response to CCL11 (eotaxin-1) was decreased in the presence of adiponectin. In contrast to the response to purified chitin and fungal conidia of the strain Af293, aspiration of the high-chitin expressing Af5517 conidia by adiponectin-deficient mice resulted in significantly increased accumulation of lung neutrophils and expression of inflammatory cytokines. Our results identify a novel role for the adiponectin pathway in inhibition of lung inflammatory responses to chitin and *A. fumigatus* inhalation.

RESULTS

Lung Inflammatory Responses to Purified Chitin Aspiration Are Associated With Decreased Lung and Serum Adiponectin and Decreased Leukocyte Surface AdipoR1 Expression

A previous study reported lung eosinophil accumulation in response to inhalation of purified chitin particles (20), and since the initial finding chitin-mediated neutrophil recruitment was also described (5). We confirmed that $CD45^{hi}Ly6G^{hi}$ neutrophils and $CD45^{hi}Ly6G^{lo}CD11c^{lo}SiglecF^{hi}$ eosinophils were markedly increased in the airways of chitin-aspirated mice, while resident $CD45^{hi}Ly6G^{lo}CD11c^{hi}SiglecF^{hi}$ alveolar macrophages were decreased (**Figures 1A,B**). In addition, inflammatory cytokines and chemokines that drive recruitment and/or activation of these cells, including IL-1α, IL-6, IL-17A, TNF, CCL11 (eotaxin-1), and CCL24 (eotaxin-2), were also increased relative to control (saline aspirated) mice (**Figure 1C**), confirming an inflammatory phenotype in the lungs of chitin-aspirated mice.

We next compared adiponectin (*adipoq*) transcript levels in lung tissue and human lung epithelial cells, and adiponectin protein levels in the serum of saline and chitin-aspirated mice. Notably, the low baseline level of lung transcription of adiponectin measured in saline-aspirated mice was significantly decreased upon aspiration of chitin particles (**Figure 2A** and data not shown), with a concomitant decrease in the concentration of serum adiponectin protein (**Figure 2B**). In contrast, curdlan (β-1,3-glucan) aspiration did not affect lung adiponectin transcription and neither chitin nor curdlan aspiration resulted in significantly altered lung leptin expression (**Figure S1**). In addition, we did not observe a consistent change in transcription of adiponectin in response to chitin in human lung epithelial (A549) cells stimulated with chitin and/or TNF (**Figure S2A**). In order to determine if fungal chitin results in decreased relative adiponectin gene expression, we compared gene expression in lungs from mice that repeatedly aspirated conidia of a normal/low chitin-expressing isolate of *A. fumigatus* (Af293) with mice that aspirated a high chitin-expressing isolate (Af5517) (21, 22). Our results indicated significantly lower adiponectin transcription in the lungs of Af5517-challenged mice when compared to repeated Af293 aspiration (**Figure 2C**). In contrast, purified chitin or swollen/fixed Af5517 conidia incubated with human lung A549 cells was not associated with a significant change in adiponectin expression, while an increase was observed in A549 cells incubated with swollen/fixed Af293 conidia (**Figure S2A**). Increased *adipoq* expression was associated with a marked increase in IL-6 mRNA and protein in response to Af293 conidia, with a lesser increase in IL-6 in response to Af5517 (**Figures S2C,D**). Thus, particulate chitin aspiration is associated with increased inflammation and decreased constitutive expression of whole lung mRNA and serum adiponectin protein, although this phenotype was not reflected in human lung epithelial A549 cells.

AdipoR1 is considered a major receptor mediating the anti-inflammatory effect of adiponectin on immune cells (10). We therefore wanted to determine if AdipoR1 was expressed on the surface of lung leukocytes and how this expression was modified by chitin aspiration. The low levels of lung eosinophils isolated from naïve mice expressed the highest relative surface staining of AdipoR1 as measured by flow cytometry, and chitin aspiration was associated with a significant decrease in wild-type B6, BALB/c and adiponectin KO (B6 background) mice (**Figures 2D,E**). Although surface expression of AdipoR1 was not as high in neutrophils and alveolar macrophages as compared to eosinophils by fluorescence intensity, AdipoR1 staining was also decreased after chitin aspiration in these populations

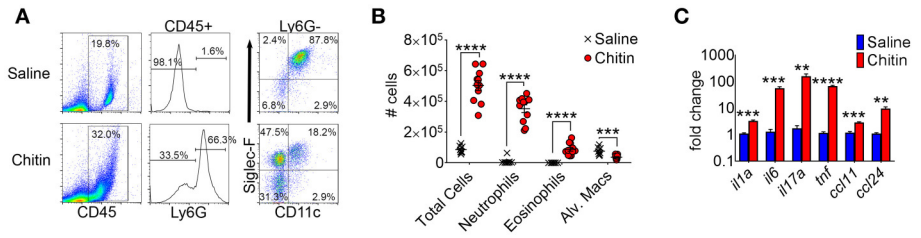

FIGURE 1 | Lung inflammatory responses after chitin aspiration. **(A–C)**, BALB/c mice were given 5 × 10^3 purified chitin particles by aspiration on consecutive days, with BALF, lung homogenates, and serum harvested for analysis 24 h after the second challenge. **(A)** Representative flow cytometric dot plots of CD45^{hi} cells from control (saline) and chitin-aspirated mice. **(B)** Total number of BALF cells, Ly6G^{hi} neutrophils, Ly6G^{lo}SiglecF^{hi}CD11c^{lo} eosinophils, and Ly6G^{lo}SiglecF^{hi}CD11c^{hi} alveolar macrophages isolated from saline or chitin-aspirated mice as determined by flow cytometry. **(C)** Quantitative RT-PCR analysis of RNA from total lung homogenates of chitin-aspirated mice. N = 6–10/group. Data are shown as a summary of 2 experiments. Error bars in all figures represent standard error of mean. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

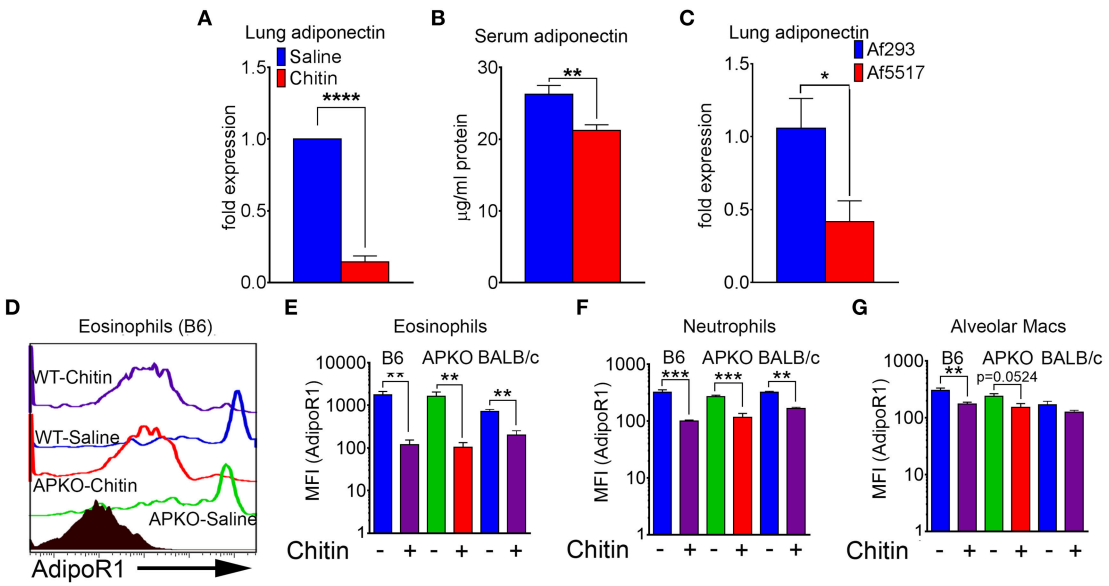

FIGURE 2 | Decreased lung, serum adiponectin and surface leukocyte AdipoR1 after chitin aspiration. **(A)** Fold change in expression of adiponectin mRNA in lung homogenates as determined by quantitative RT-PCR. N = 6–10/group. **(B)** Concentration of serum adiponectin protein in saline and chitin-aspirated mice as determined by ELISA. N = 5–6/group. **(C)** Fold expression of lung adiponectin gene expression in BALB/c mice that repeatedly aspirated 2 × 10^6 conidia of low/normal (Af293) or high (Af5517) chitin-expressing isolates. **(D–G)** BALF was harvested and eosinophils were analyzed by flow cytometry for AdipoR1 expression. **(D)** Representative histograms of AdipoR1 staining on eosinophils. **(E–G)** Summary of median fluorescence intensities of AdipoR1 staining on eosinophils, neutrophils, and alveolar macrophages from 2 experiments with wild-type or adiponectin-deficient mice, B6 background, or wild-type mice, BALB/c background. Data are a summary of 2–3 experiments. N = 6–10/group. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

(**Figures 2F,G**). In A549 cells, *adipoR1* transcription was not significantly modulated in response to chitin or swollen/fixed conidia (**Figure S2B**). Thus, chitin aspiration may result in inhibited responsiveness to adiponectin by decreased surface AdipoR1 in multiple lung leukocyte populations.

Previous studies showed that transgenic lung airway expression of acidic mammalian chitinase (SPAM) inhibited chitin-mediated recruitment of eosinophils and M2 macrophage activation (20, 22, 23). We wanted to determine if chitin degradation in chitin-aspirated SPAM mice resulted in increased lung adiponectin transcription compared to non-transgenic mice. We observed a consistent decrease in lung mRNA from the genes that encode IL-1a, IL-6, IL-17A, TNF, CCL11, and CCL24 in SPAM+ mice compared to SPAM- mice (**Figure 3A**). In contrast, adiponectin gene expression was not significantly altered. Total lung cells were decreased in SPAM+ mice (**Figure 3B**), mainly reflected by decreased neutrophils (**Figure 3C**). Eosinophils in SPAM+ mice were not significantly decreased (**Figure 3D**), while alveolar macrophages were increased in transgenic animals (**Figure 3E**). Although inflammatory responses were significantly inhibited with transgenic expression of lung AMCase, enhanced enzymatic chitin degradation was not associated with a concomitant increase in lung adiponectin gene expression.

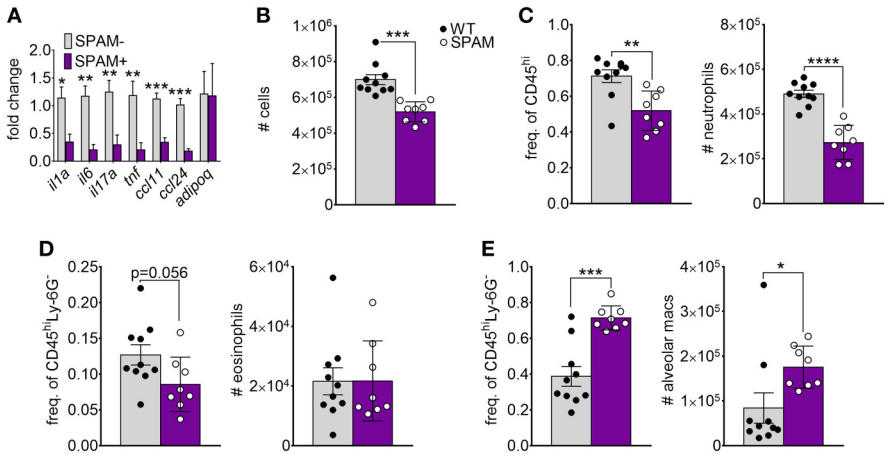

FIGURE 3 | Decreased chitin-induced inflammation with transgenic expression of lung AMCase. SPAM transgenic mice or non-transgenic littermates were given chitin particles by involuntary aspiration as described for **Figure 1**. **(A)** RNA from lung homogenates was analyzed by qRT-PCR for the indicated cytokines. N = 6–10/group. **(B–E)** Total BALF leukocytes **(B)**, frequency (left panels) and total numbers (right panels) of neutrophils **(C)**, eosinophils **(D)**, and alveolar macrophages **(E)** as determined by flow cytometry. Data are a summary of 2 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Inhibition of Chitin-Mediated Eosinophil Recruitment by Adiponectin

Since chitin aspiration is associated with decreased adiponectin in addition to inducing lung accumulation of eosinophils, we also wanted to determine if adiponectin inhibits chitin-mediated eosinophil and/or neutrophil accumulation. In order to accomplish this, we first co-aspirated BALB/c mice with recombinant murine adiponectin (0.5 μg/Kg) along with chitin particles and compared eosinophil recruitment to mice that aspirated adiponectin or chitin alone. Although numbers of total leukocytes, neutrophils, and alveolar macrophages remained unchanged with adiponectin co-aspiration (**Figures 4A–C**), airway eosinophils were significantly decreased with co-aspiration compared to mice that only aspirated purified chitin (**Figure 4D**). Despite this reduction in eosinophils, we did not observe a significant decrease in the transcripts of inflammatory cytokine genes or the eosinophil-attracting chemokines CCL11 and CCL24 (**Figure 4E**). Reciprocally, when mice deficient in adiponectin aspirated chitin particles, airway eosinophils, but not other leukocytes, were increased in comparison to wild-type C57BL/6 mice (**Figures 4F–I**), although the relative numbers of airway eosinophils were lower in B6 background mice compared to BALB/c mice (**Figure 4D**). Lung *ccl11* mRNA was increased in chitin-aspirated, adiponectin-deficient mice, while *il1a*, *il6*, *tnf*, and *ccl24* were not significantly altered, and *il17a* mRNA was decreased (**Figure 4J**). Thus, administration of exogenous adiponectin resulted in a specific decrease in chitin-mediated lung eosinophil accumulation with increased eosinophils in the absence of adiponectin.

We also compared chemotaxis of bone marrow-derived eosinophils (BMEs) in response to CCL11 (eotaxin-1) in the presence or absence of adiponectin, verifying the BME phenotype by microscopy (**Figure 5A**) and flow cytometry/qRT-PCR for surface expression of Siglec-F and/or AdipoR1/R2 (**Figures 5B,C**). Pre-incubation with recombinant adiponectin resulted in decreased transwell migration of BMEs in response to CCL11 (**Figure 5D**). Therefore, our results from multiple approaches suggest that adiponectin inhibits chitin-mediated eosinophil recruitment and migration.

Fungal Isolate-Specific Increase in Lung Neutrophil Recruitment and Inflammatory Cytokine Expression in Adiponectin-Deficient Mice

In *A. fumigatus*, chitin is a cell wall structural molecule that is covalently linked with other immune-stimulating molecules like β-1,3-glucan that are co-exposed on the surface of swollen, germinating conidia, and hyphae (24). Thus, innate recognition of chitin in response to fungal inhalation and infection occurs with co-recognition of other fungal PAMPs. We previously reported that immune responses to the high chitin-expressing *A. fumigatus* isolate Af5517 were skewed toward type 2 immune responses with increased eosinophil recruitment (22). Thus, we wanted to compare the response to aspiration of conidia from the Af293 and Af5517 isolates in the absence of adiponectin. In contrast to the unchanged response to the normal/low chitin-expressing Af293 isolate in wild-type and adiponectin deficient mice, aspiration of viable Af5517 conidia resulted in increased neutrophil recruitment with decreased alveolar macrophages compared to wild-type mice in the absence of adiponectin (**Figures 6A,B**), while eosinophils were not significantly changed in response to either isolate (**Figure 6C**). Furthermore, alveolar macrophages were decreased in the absence of adiponectin in response to Af5517 (**Figure 6D**). Consistent with increased inflammation, *il1a*, *il6*, *il17a*, and *ccl24* lung mRNA levels were increased in adiponectin KO mice in response to Af5517, but not Af293 conidia (**Figure 6E**). Our results demonstrate a fungal

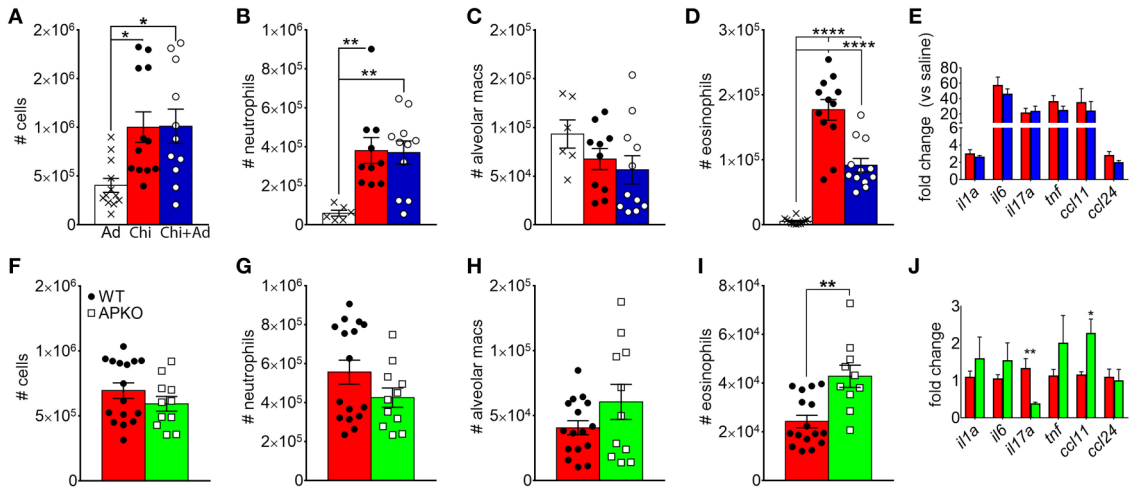

FIGURE 4 | Inhibition of chitin-mediated airway eosinophil recruitment by adiponectin. BALB/c **(A–E)** or C57BL/6 wild-type (WT) or adiponectin-deficient (APKO) **(F–J)** mice aspirated chitin particles as described for **Figure 1**, with BALF analyzed by flow cytometry 24 h after the second challenge. **(A–E)** Mice aspirated 0.5 μg/Kg recombinant adiponectin alone, chitin particles alone, or particles + adiponectin. **(A,F)** Total CD45hi leukocytes, **(B,G)**, neutrophils. **(C,H)**, alveolar macrophages **(D,I)**, eosinophils. **(E,J)** Quantitative RT-PCR of lung homogenate RNA to determine expression of the indicated cytokines/chemokines. N = 6–10/group. Data are a summary of 2–3 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

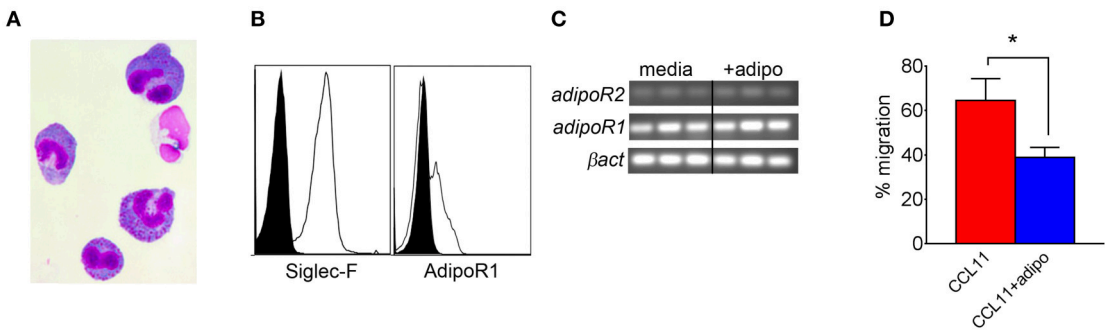

FIGURE 5 | Decreased bone marrow-derived eosinophil migration in response to CCL11 in the presence of adiponectin. Bone marrow-derived eosinophils (BMEs) were characterized by microscopy for morphology **(A)**, flow cytometry for surface expression of Siglec-F and *adipoR1* **(B)**, RT-PCR for transcription of *AdipoR1* and *adipoR2* **(C)**, and with a transwell migration inhibition assay in response to recombinant CCL11 in the presence of absence of recombinant adiponectin, with three replicates **(D)**. Data are representative of two experiments with similar results. $^{*}p < 0.05$.

isolate-specific increase in lung inflammation in adiponectin-deficient mice in response to aspiration of *A. fumigatus* conidia with a distinct profile in comparison to responses to purified chitin.

DISCUSSION

In this study, our initial goal was to determine if adiponectin inhibited lung chitin-mediated eosinophil recruitment, since others had shown that adiponectin inhibited allergic lung inflammation and eosinophil migration and adhesion (17, 25, 26). For this we used an established model of lung eosinophil recruitment that involves inhalation of purified chitin (**Figure 1**). We used a commercially prepared purified large chitin particle suspension (<70 μm) due to the established effect on eosinophil recruitment, despite differences in size and acetylation with purified fungal chitin particles (2). In mice that aspirated purified chitin, we observed that expression of lung adiponectin and adiponectin receptor AdipoR1 on eosinophils, neutrophils, and alveolar macrophages were decreased (**Figure 2**). However, our results with human lung epithelial A549 cells did not display similar changes in adiponectin or AdipoR1 gene expression in response to chitin or fungal conida (**Figure S2**). Increased constitutive lung chitinase activity in SPAM transgenic mice was sufficient for decreased lung inflammatory cytokines and leukocytes in comparison to non-transgenic littermates, but was not sufficient for increased adiponectin transcription (**Figure 3**). Mouse aspiration of recombinant adiponectin resulted in a specific decrease in lung chitin-mediated eosinophil recruitment, while lung

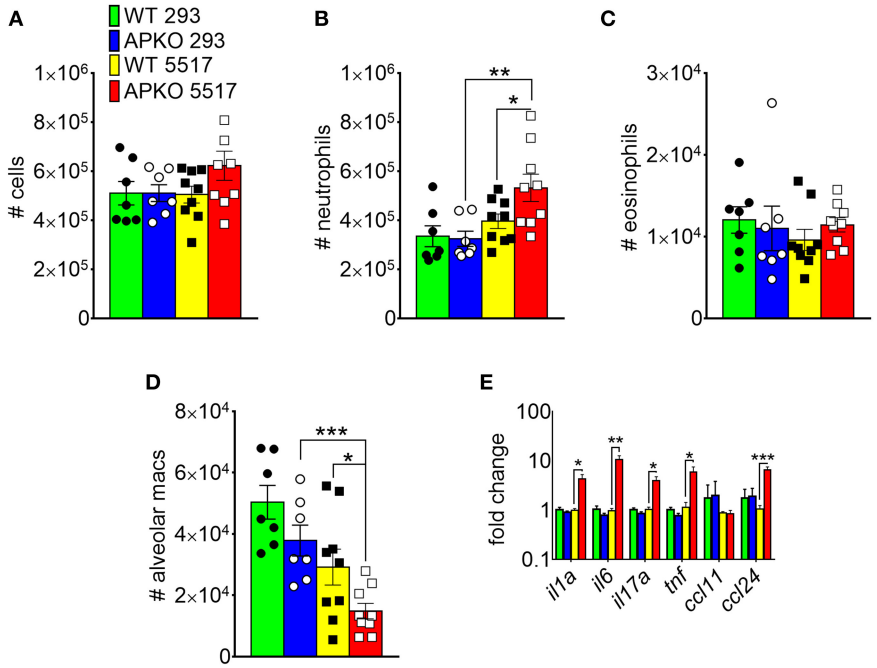

FIGURE 6 | Fungal isolate-specific modulation of airway leukocyte recruitment and increased lung inflammatory cytokine expression in adiponectin-deficient mice in response to *A. fumigatus* aspiration. Wild-type or adiponectin-deficient mice aspirated 5×10^6 conidia from Af293 or Af5517 isolates; lungs were harvested 48 h post-challenge for analysis of BALF cells for the indicated cell types by flow cytometry **(A-D)** or lung homogenate cytokine mRNA by qRT-PCR **(E)**. $N = 6$–10/group. Data are a summary of two independent experiments for each fungal isolate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inflammatory cytokines and chemokines were not significantly modulated (**Figures 4A–D**). Reciprocally, eosinophils and the corresponding eosinophil chemokine CCL11 (eotaxin-1) were increased in adiponectin-deficient mice compared with wild-type (**Figure 4I**). The presence of recombinant adiponectin also resulted in decreased bone marrow-derived eosinophil migration in response to CCL11 (**Figure 5**). Thus, our results from multiple approaches collectively support a role for adiponectin in inhibition of chitin-mediated eosinophil recruitment, and suggest a reciprocal chitin-driven decrease in lung adiponectin and adiponectin receptor expression.

Although our results were consistent with an inhibitory role for adiponectin in eosinophil recruitment and migration in response to purified chitin, adiponectin is also known to inhibit activation and/or cytokine secretion of NK cells, neutrophils, dendritic cells, and γδ T cells (10). Although we did not see decreased recruitment of neutrophils and alveolar macrophages in the presence of adiponectin, AdipoR1 expression on these cells was still relatively decreased after chitin aspiration compared to cells from naïve animals (**Figures 2F,G**). This suggests that other adiponectin-regulated cellular processes could be decreased by chitin aspiration in these cells. Lung macrophages and γδ T cells may also be activated by particulate chitin inhalation (8, 20). Although we did not observe changes in adiponectin or adipoR1 mRNA in lung epithelial A549 cells in response to chitin or fungal conidia (**Figure S2**), other groups have also reported AdipoR1 expression in these cells as well as inhibition of cell proliferation and viability in the presence of adiponectin (27–30). Thus, macrophages, γδ T cells, and lung epithelial cells are interesting targets for future studies to determine cell-specific modulation of fungal responses by adiponectin.

Aspiration of the fungal conidia isolate Af5517, which expresses increased chitin covalently linked with other immune-stimulating fungal PAMPs such as β-1,3-glucan (21), resulted in increased lung neutrophil recruitment and inflammatory cytokine gene expression in adiponectin-deficient mice, whereas Af293 aspiration had no significant difference (**Figures 6B,E**). In contrast with purified chitin, viable Af5517 conidia aspiration did not result in significantly increased eosinophil recruitment (**Figure 6C**). Initially, this appears to contradict our previous results (22). However, in the previous study, swollen and fixed Af5517 conidia were reported with significantly increased eosinophil recruitment after a single aspiration, whereas type 2 immune responses and eosinophil recruitment were markedly elevated after multiple aspirations of viable conidia. The differences in airway eosinophil recruitment after a single aspiration might be due to differences in temporal exposure of fungal PAMPs and concomitant immune recognition of viable vs. swollen/fixed conidia in both models. Furthermore, it is certain that Af293 and Af5517 exhibit phenotypic differences beyond cell wall composition, and thus differences between the strains can only be correlated with immune responses. Despite these caveats, we believe our data with particulate chitin and fungal conidia are consistent with broad targets for a regulatory action of adiponectin, especially in response to the combined

stimulation of multiple PAMPs presented by germinating *A. fumigatus* conidia. Our future studies will examine the effects of adiponectin and roles of adiponectin receptors on the modulation of activation and inflammatory cytokine secretion in multiple cells types in response to co-recognition of multiple fungal PAMPs.

The global obesity epidemic and the association of obesity with a heightened inflammatory state has ignited widespread interest in the mechanisms of systemic and cellular immunometabolism (31). Many recent studies have focused on the effect of immune pathways in the regulation of adipose tissue and systemic metabolic homeostasis (31, 32). However, this relationship is reciprocal, as adipose tissue cytokines/hormones that were initially characterized as regulators of systemic metabolism, including adiponectin, are also known to play roles in immune regulation (33). Interestingly, we observed that chitin aspiration decreased serum levels of adiponectin and adipose tissue eosinophils (**Figure 2D** and unpublished data), suggesting that lung recognition of chitin affects metabolic and immune regulation at distal sites. Interestingly, a recent study reported that obese or adiponectin-deficient mice displayed decreased clearance of *Listeria monocytogenes* due to inhibition of chronic bone marrow inflammation (19). Furthermore, our results from a parallel study suggest the adiponectin dampens detrimental inflammation in invasive pulmonary aspergillosis (Amarsaikhan et al., submitted). Detrimental inflammation is also an important factor in allergic bronchopulmonary aspergillosis (ABPA), where lung airway persistence/colonization in susceptible individuals leads to inflammatory pathology mediated in part by eosinophils (34). However, the role of adiponectin in protection from ABPA remains unknown. In future studies, we aim to further explore the novel role for this pathway in dampening inflammation and improving survival in models of fungal inhalation, airway colonization, and invasive infection.

MATERIALS AND METHODS

Mouse Strains

BALB/c, C57BL/6, and adiponectin-deficient (*adipoq*-/-) mice (B6 background) were received from Jackson Laboratory. SPAM transgenic mice with constitutive expression of acidic mammalian chitinase (AMCase) under the lung Clara cell-specific promoter *cc10* were provided by Dr. Richard Locksley [University of California, San Francisco (20)].

Growth and Handling of Fungi

The clinical isolate Af293 was previously obtained from the Fungal Genetics Stock Center. The *A. fumigatus* isolate Af5517 was obtained from the United States Agriculture Research Service. Fungi were grown on Malt Extract Agar plates at 22°C and conidia suspensions were collected and aseptically prepared as described (21, 22).

Mouse Aspiration of Chitin or Fungal Conidia

Custom-sized purified chitin particles (<70 μm) were obtained from New England Biolabs and prepared as previously described (20). Purified chitin particle suspensions were delivered by involuntary aspiration of 50 μl solution to isoflurane-anesthetized mice. For particles, 100 particles/μl were aspirated daily for 2 days and mice were sacrificed 24 h after final challenge to assess innate immune responses. For the adiponectin co-aspirations with chitin particles, recombinant adiponectin (Sino Biologicals) was reconstituted to the optimal concentration of 0.25 μg/Kg (x2 for a total of 0.5 μg/Kg) in Phosphate Buffered Saline (PBS) and aspirated into the airway alone or in combination with chitin particles. For repeated aspiration of conidia, 2×10^6 conidia were aspirated and mice were sacrificed 72 h after the final challenge to assess inflammation and T cell-mediated responses as previously described (22). For single aspiration of conidia, 5×10^6 Af293 or Af5517 conidia were aspirated and mice were harvested 48 h later to assess both neutrophil and eosinophil recruitment as well as inflammatory cytokine transcription (22, 35).

Sample Collection and Processing

For transcription quantification, mouse lungs were harvested, flash frozen and used for total RNA extraction and analysis as previously described (22). Primers for qRT-PCR were obtained from SABiosciences. Serum was separated from blood collected by cardiac puncture and serum adiponectin levels were measured by ELISA according to manufacturer's instructions (R&D Systems). All flow cytometry reagents were obtained by BD biosciences or eBioscience, with the following exception: Rabbit mAb for mouse AdipoR1 was used along with IgG1 isotype control for primary stain followed by Goat anti-rabbit IgG Dylight 488 secondary antibody stain (both from Abcam). Populations of cells were evaluated by flow cytometric analysis on a Guava EasyCyte 8HT bench top flow cytometer (EMD Millipore) as previously described (22). For color compensation, mouse splenocytes were left unstained or stained with single color controls of rat anti-mouse CD4 antibodies.

In vitro Eosinophil Culturing From Bone Marrow

Bone marrow derived eosinophil cultures were generated with bone marrow cells isolated from femurs of BALB/c mice, followed by incubation with recombinant SCF, FLT3L, and IL-5 as described by Dyer et al. (36). Before use in experiments, differentiated eosinophils were confirmed using cytospin followed by with histology staining (DiffQuick) and flow cytometry for Siglec-F expression. Mature eosinophils after Day 12 were enumerated and used for chemotaxis assay and total RNA isolation. For chemotaxis assay, eosinophils were incubated with 5 μg/ml recombinant adiponectin (R&D) for 60 min. Migration was measured by counting cells on hemocytometer after cells were incubated on transwell (5 μm pore size, Costar) with bottom chamber media containing 100 ng/ml eotaxin. Positive control with eotaxin-1 (CCL11) in both chambers and negative control with no chemokine was included. For gene expression of *adipoR1* and *adipoR2* total RNA was isolated from cells treated with and without adiponectin for 4 h. Total RNA isolation was performed with Trizol (Ambion) method

combined with RNAeasy mini purification columns according to manufacturer's protocol (Qiagen).

Lung Epithelial Cell Culture

The human lung epithelial cell line A549 was obtained from ATCC and cultured according to supplier's protocol in F-12K media with 10% FBS at 37°C with 5% CO_2. For experiments, 1×10^6 cells/ml were seeded overnight in no serum growth media to arrest growth. Next day, media was replaced with regular serum media with or without 100 ng/ml recombinant human TNFα (R&D Systems) [to induce adiponectin expression as previously described (27)] alone or co-incubated with purified chitin particles (5,000 particles/ml) or 1×10^7 Af293 or Af5517 conidia for 6 h (MOI 10). Post incubation, media was removed and cells were lysed in 1 ml Trizol. Total RNA was isolated with Qiagen RNAeasy columns, cDNA synthesized and used for qRT-PCR for gene expression analysis. Secreted cytokines from cell free culture supernatants were quantified using ELISA kits (Peprotech) according to manufacturer's protocols.

Data Analysis Methods

Analysis of mouse flow cytometric data was performed with FlowJo software, version 10 (Becton-Dickinson). Prism 6 software was used for generation of graphs and figures and for statistical analyses (GraphPad). Unpaired *t*-tests were used to measure statistical significance when two groups were directly compared, and one or two-way analysis of variance (ANOVA) tests were used for comparison of three or more groups, followed by Tukey's or Sidak's post-tests for multiple comparisons, respectively. Differences between experimental groups that resulted in a $p < 0.05$ were considered significant.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the PHS Policy on Humane Care and Use of Laboratory Animals. The protocol was approved by the Indiana State University Animal Care and Use Committee, the host campus of IUSM-Terre Haute.

AUTHOR CONTRIBUTIONS

ST conceived the project and wrote the paper. ST and NA designed the experiments. NA, DS, AW, ES, AT, and HG performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01057/full#supplementary-material

Figure S1 | Lung leukocyte recruitment, adiponectin, and leptin transcription 24 h after a single aspiration with 3 mg curdlan (in 50 μl saline). **(A)** Total cells, **(B)** neutrophils, **(C)** eosinophils, **(D)** adiponectin (adipoq) expression by qRT-PCR (Saline control baseline). **(E)** Leptin expression in saline, curdlan, and chitin-aspirated mice. **(D,E)** $N = 4$/group. ***$p < 0.001$. ****$p < 0.0001$.

Figure S2 | Adiponectin, AdipoR1, and selected cytokine expression in human lung epithelial A549 cells in response to purified chitin or *A. fumigatus* conidia. A549 cells were incubated for 6 h with or without human recombinant TNF and/or purified chitin, or swollen/fixed *A. fumigatus* conidia from Af293 or Af5517 isolates as described in Materials and Methods. **(A–C)** Messenger RNA quantification by qRT-PCR of the indicated genes in cell lysates. **(D)** Quantification of protein levels of indicated cytokines from cell supernatants by ELISA. Data are a summary of two experiments with $N = 6$/group. *$p < 0.05$. **$p < 0.01$. ****$p < 0.0001$.

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